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Development of a novel high retention enzymatic membrane bioreactor (HR-EMBR) for the removal of trace organic contaminants

Muhammad Bilal Asif
University of Wollongong

School of Civil, Mining and Environmental Engineering
Faculty of Engineering and Information Sciences
University of Wollongong, Wollongong, Australia

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Certification

I, Muhammad Bilal Asif, hereby declare that this thesis, submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy to the School of Civil, Mining and Environmental Engineering, Faculty of Engineering and Information Sciences, University of Wollongong, is wholly my own work unless otherwise acknowledged. The document has not been submitted for qualification at any other academic institution.

Muhammad Bilal Asif

Thesis-related Publications

Journal articles

1. **Asif, M.B.**, Hai, F.I., Dhar, B.R., Ngo, H.H., Guo, W., Jegatheesan, V., Price, W.E., Nghiem, L.D., Yamamoto, K. 2018. Impact of simultaneous retention of micropollutants and laccase on micropollutant degradation in enzymatic membrane bioreactor. *Bioresource Technology, 267, 473-480*.
2. **Asif, M.B.**, Hai, F.I., Kang, J., Van De Merwe, J.P., Leusch, F.D., Price, W.E., Nghiem, L.D. 2018. Biocatalytic degradation of pharmaceuticals, personal care products, industrial chemicals, steroid hormones and pesticides in a membrane distillation-enzymatic bioreactor. *Bioresource Technology, 247, 528-536*.
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2. **Asif, M.B.**, Hai, F.I., Jegatheesan, V., Price, W.E., Nghiem, L.D., Yamamoto, K. 2019. Applications of Membrane Bioreactors in Biotechnology Processes. in: *Current Trends and Future Developments on (Bio-) Membranes: Membrane processes in the pharmaceutical and biotechnological field*, (Eds.) A. Basile, C. Charcosset, Elsevier. Netherlands, pp. 223-257. (ISBN: 9780128136065)

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Additional-relevant Publications

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Book chapters

1. **Asif, M.B.**, Hai, F.I., Price, W.E., Nghiem, L.D. 2018. Impact of pharmaceutically active compounds in marine environment on aquaculture. in: *Sustainable Aquaculture*, (Eds.) F.I. Hai, C. Visvanathan, R. Boopathy, Springer. Switzerland, pp. 265-299. (ISBN: 9783319732565)
2. **Asif, M.B.**, Hai, F.I. 2019. Wastewater reuse applications and MBR. in: *Membrane Biological Reactors: Theory, Modeling, Design, Management and Applications to Wastewater Reuse (2nd Edition)*, (Eds.) F.I. Hai, K. Yamamoto, C. Lee, IWA publishing. United Kingdom pp. 183-206. (ISBN: 9781780409177)
3. Hai, F.I., Nghiem, L.D., Khan, S.J., **Asif, M.B.**, Price, W.E., Yamamoto, K. 2019. Removal of emerging trace organic contaminants (TrOC) by MBR. in: *Membrane Biological Reactors: Theory, Modeling, Design, Management and Applications to Wastewater Reuse (2nd Edition)*, (Eds.) F.I. Hai, K. Yamamoto, C. Lee, IWA publishing. United Kingdom pp. 413-468. (ISBN: 9781780409177)
4. **Asif, M.B.**, Hai, F.I. 2019. MBR design calculations. in: *Membrane Biological Reactors: Theory, Modeling, Design, Management and Applications to Wastewater Reuse (2nd Edition)*, (Eds.) F.I. Hai, K. Yamamoto, C. Lee, IWA publishing. United Kingdom. (ISBN: 9781780409177)

Abstract

This thesis systematically investigates the performance of a novel high retention (HR) – enzymatic membrane bioreactor (EMBR) for effective degradation of a broad spectrum of trace organic contaminants (TrOCs) commonly detected either in sewage-impacted water or in wastewater treatment plant effluent. In the last decade, laccase (EC 1.10.3.2), a copper-containing oxidoreductase enzyme, has been studied extensively for the degradation of recalcitrant pollutants. Laccase-catalysed degradation of TrOCs such as pharmaceuticals, pesticides, personal care products, industrial chemicals and steroid hormones has gained significant attention. These TrOCs occur ubiquitously in municipal wastewater and sewage-impacted water bodies. This can potentially be harmful to aquatic ecosystems and human health.

Initially, performance of laccase was assessed in batch enzymatic bioreactors due to the concern of enzyme washout in a continuous-flow treatment system. In an attempt to prevent enzyme washout, an enzymatic membrane bioreactor (EMBR) was developed by coupling an ultrafiltration (UF) membrane to an enzymatic bioreactor. Interestingly, during the operation of the EMBR, adsorption of some hydrophobic TrOCs (log D >3) onto the enzyme gel layer over the membrane surface resulted in enhanced degradation of the adsorbed compounds. This observation indicates the complementarity of simultaneous laccase and TrOC retention within EMBR. Hence, in this thesis, a novel high retention enzymatic membrane bioreactor (HR-EMBR) system was developed for enhanced TrOC degradation by coupling an enzymatic bioreactor with a high retention membrane separation process such as nanofiltration (NF) or membrane distillation (MD).

To serve as the proof of concept, the first step of this research compared the performance of a conventional UF- and high retention NF- EMBRs for the degradation of a broad spectrum of 29 TrOCs under identical operating conditions such as TrOC-loading rate and hydraulic retention time. The results revealed that the overall removal (*i.e.*, biodegradation + membrane retention) of TrOCs in NF-EMBR was better as compared to that achieved by UF-EMBR. This is because the NF membrane achieved TrOC rejection ranging from 90 to 99%. Furthermore, mass balance analysis shows that, as compared to the UF-EMBR, significantly better degradation (up to 65%) was achieved by laccase in NF-EMBR. Improved degradation following simultaneous TrOC and laccase retention was mainly due to the prolonged contact time. Formation of secondary radicals or coupling agents, which are formed following laccase-catalysed degradation of phenolic TrOCs, are highly reactive and could directly oxidize or polymerize other TrOCs. Notably, the results of this study suggest that UF membrane can contribute to the removal of TrOCs depending on their hydrophobicity and charge, thereby improving the overall performance of UF-EMBR. The overall removal by the NF-EMBR was considerably better due to enhanced TrOC degradation as well as effective TrOC removal.

Permeate flux of UF/NF membranes reduced gradually, this can be attributed to: (i) membrane fouling due to the adsorption of laccase on membrane surface forming an enzyme gel-layer; and/or (ii) concentration polarization due to the accumulation of TrOCs and transformation products on membrane surface. Membrane cleaning with water was suitable for effective flux recovery.

In vitro treatment with laccase mainly depends on two factors: (i) redox-potential; and (ii) availability of electron donating (EDG) or withdrawing (EWG) functional groups in the chemical structure of TrOCs. Depending on its source, the catalytic potential of laccase for TrOC removal may significantly vary. In the next part of this research work, efficacy of two different laccases from genetically modified *Aspergillus oryzae* and *Trametes versicolor* was analysed in by coupling an enzymatic bioreactor with the MD process, which is another format of high retention membrane. Following effective TrOC retention (>99%) by the MD membrane, enhanced laccase-catalysed degradation of the selected TrOCs was achieved in MD-EMBR as compared to previously developed UF-EMBRs. Importantly, although degradation by both laccases was TrOC-specific, performance of laccase from *A. oryzae* was superior to that obtained by laccase from *T. versicolor*. This could be attributed to the higher redox-potential (up to 15%) of laccase from *A. oryzae* than laccase from *T. versicolor*.

Although MD-EMBR can produce TrOC-free permeate, enzymatic degradation of certain groups of TrOCs (*e.g.,* those containing strong EWGs) was incomplete. The spectrum of efficiently degraded TrOCs can be extended by introducing a naturally occurring or synthetic redox-mediator that acts as an electron shuttle between the TrOCs and laccase. Hence, the performance of three redox-mediators, namely syringaldehyde, violuric acid and 1-hydroxybenzotriazole was assessed for improving the degradation of TrOCs in MD-EMBR. Each redox-mediator achieved TrOC-specific improvement in degradation, but violuric acid was the most efficient and versatile redox-mediator. However, when a mixture of syringaldehyde and violuric acid was tested, instead of inducing a synergistic effect, degradation of at least six pharmaceutically active TrOCs reduced. Despite the improved TrOC degradation, a mediator-specific increase in toxicity of bioreactor media as well as rapid laccase inactivation was observed following their addition in the enzymatic bioreactor of MD-EMBR. Nevertheless, the effluent of the MD-EMBR (*i.e.*, membrane permeate) was non-toxic. This is because the high retention MD membrane could retain all the constituents of enzymatic bioreactor.

To address the issue of laccase inactivation in presence of redox-mediators, an integrated persulfate- and laccase- based oxidation process was envisioned. Based on the results achieved in batch tests, effect of persulfate concentration (1-10 mM) and incubation time (up to 24 h) as well as persulfate activation pathways were elucidated. The results revealed that the combined laccase/persulfate-assisted oxidation process achieved improved degradation of TrOCs resistant to laccase only. The developed process was also effective for estrogenicity reduction

without causing significant laccase inhibition. A NF membrane when coupled with the laccase/persulfate-assisted oxidation process allowed continuous-flow operation. This is because NF membrane effectively retained both persulfate and laccase. Importantly, degradation of non-phenolics further improved by 10 to 65% in laccase/persulfate-NF system as compared to laccase only. This could be attributed to the prolonged contact time between laccase/PS and TrOCs; as well as the contribution of oxidative coupling agents in degradation. The toxicity and estrogenicity bioassays confirmed that membrane permeate was non-toxic and safe for disposal.

Physicochemical properties of raw water collected from surface water and groundwater are diverse. Raw water matrix contains different dissolved organic and inorganic (*e.g.*, metal ions) impurities. The freshwater bodies may contain both TrOCs and metal ions (*e.g.*, iron) due to sewage contamination as well as acid mine drainage (AMD) intrusion. Different treatment options including the stand-alone and integrated MD system were examined for efficient treatment of sewage- and AMD-impacted water. The stand-alone MD system successfully retained (85-100%) bulk organics, TrOCs and metal ions (iron, magnesium, calcium and lithium). However, accumulation of organics and metal ions caused severe membrane fouling, consequently reducing the permeate flux by up to 75% within 5 d of operation. Based on the performance of laccase and persulfate in batch tests, a PS-assisted oxidation process was selected to be coupled with the MD system. The PS-MD system reduced the accumulation of bulk organics and TrOCs, but membrane scaling mainly caused by iron still affected permeate flux. Nevertheless, the MD membrane effectively retained all impurities, and consistently produced pollutant-free permeate (*i.e.*, treated effluent).

Keywords: Biodegradation; Effluent toxicity; Enzymatic membrane bioreactor; Estrogenicity; High retention membranes; Laccase; Membrane distillation; Membrane fouling; Nanofiltration; Trace organic contaminants (TrOCs)

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Muhammad Bilal Asif
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List of Abbreviations

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ADI	Acceptable daily intake
ALR	Anticonvulsant and Lipid regulator
AMD	Acid mine drainage
AOPs	Advanced oxidation processes
AR	Artificial recharge
CAS	Conventional activated sludge
DCMD	Direct contact membrane distillation
DEET	N, N-Diethyl-meta-toluamide
DMP	2,6-dimethoxyphenol
DO	Dissolved oxygen
E1	Estrone
E2	17β-Estradiol
E3	Estriol
EDCs	Endocrine disrupting chemicals
EDGs	Electron donating functional groups
EE2	17α-Ethynylestradiol
EMBR	Enzymatic membrane bioreactor
EQS	Chronic environmental quality standards
EWG	Electron withdrawing functional groups
FO	Forward osmosis
GAC	Granular activated carbon
GC-MS	Gas chromatography- mass spectrometry
HBT	1-hydroxylbenzotriazole
HPLC	High performance liquid chromatography
HR-EMBR	High retention enzymatic membrane bioreactor
HR-MBR	High retention membrane bioreactor
HRT	Hydraulic retention time
LC-MS	Liquid chromatography- mass spectrometry
LiP	Lignin peroxidase

Log D	Water partitioning coefficient
MBR	Membrane bioreactor
MD	Membrane distillation
MnP	Manganese peroxidase
MWCO	Molecular weight cut off
NF	Nanofiltration
NSAIDs	Nonsteroidal anti-inflammatory drug
PCPs	Personal care products
PhACs	Pharmaceutically active compounds
PNEC	Predicted no-effect concentration
PPCPs	Phamaceuticals and personal care products
PS	Persulfate
RO	Reverse osmosis
RQ	Risk quotient
SA	Syringaldehyde
SEM-EDS	Scanning electron microscopy – Energy dispersive X-ray spectroscopy
SPE	Solid phase extraction
TEMPO	2,2,6,6-tetramethylpiperidin-1-yloxy
TN	Total nitrogen
TOC	Total organic carbon
TrOCs	Trace organic contaminants
UF	Ultrafiltration
VA	Violuric acid
WRF	White-rot fungi
WWTPs	Wastewater treatment plants

Chapter 1: Introduction

1.1. Background

Due to the rapid population increase and urbanization, water demand for domestic, industrial and agricultural activities is increasing at an alarming rate [7, 8]. Less than 3% of all the water on earth is categorized as freshwater, most of it frozen in glaciers, ice and snow, and less than 1% present as fresh groundwater and soil moisture. Less than 0.01% of it is present as surface water in lakes, swamps and rivers. The situation is exacerbated by erratic rainfall patterns due to climate change, which impose new challenges to the already water-stressed areas [8-10]. In addition to effective water resource management, wastewater treatment and reuse are important strategies because wastewater can serve as an alternative non-conventional source of water for diverse end-user applications (*e.g.*, irrigation and non-potable reuse), particularly in water scarce regions. The concept of water reuse dates back the 1920s [11-13]. Given the ever-increasing water demand and uncertain freshwater supply, the importance of water reuse cannot be overstated [14, 15]. Many countries around the world either have established stringent regulations to ensure water reuse or have initiated programs to promote water reuse. For instance, a few states of USA such as California, Florida and Washington have adopted regulations for mandatory connection to the reclaimed water network, if available [16], thus creating opportunities for investment in future water reuse projects. Importantly, the financial model of water reuse projects continue to evolve for attracting investments [17]. These projects are also eligible for subsidies [16]. For example, water recycling projects in Australia has received approximately $800 million in funding/subsidies from Water Smart Australia program initiated by Australian Government [18]. The market for advanced water reuse (*i.e.*, direct and indirect potable) is growing rapidly in USA, Australia, Mexico, China, Spain and Saudi Arabia [14], and the total market is expected to reach $12 billion by 2025 [8, 19].

In the last decade, water reclamation and reuse have received particular attention to meet water demand during long term droughts and to improve and strengthen water supply portfolio. For safe water reuse applications, effective removal of a wide range of pollutants including bulk organics, salts, nutrients and trace organic contaminants (TrOCs) is essential. Among these pollutants, the effective removal of TrOCs is one of the most challenging aspects of wastewater treatment and reuse as conventional activated sludge (CAS)-based wastewater treatment plants were not designed for their removal [20, 21].

TrOCs include a diverse group of chemicals such as pharmaceuticals, ingredients of personal care products, endocrine disrupting chemicals (EDCs), pesticides and industrial chemicals. Most

groups of TrOCs such as pharmaceuticals and ingredients of personal care products are of municipal origin and are used in homes and workplaces on daily basis, leading to their widespread occurrence in municipal wastewater [22-24]. A few other groups of TrOCs such as pesticides can contaminate municipal wastewater and freshwater bodies *via* leaching from roads and parks/gardens during rainfall events [23, 25]. According to a thorough literature survey, wastewater is the main source of TrOC occurrence in freshwater, hence, their removal in conventional wastewater treatment plants (WWTPs) is of prime significance [24, 26]. Conventional WWTPs cannot efficiently remove certain groups of TrOCs such as antibiotics, pesticides and some industrial chemicals [21, 27]. Presence of TrOCs in the treated effluent could cause severe ecological health concerns even at a trace concentration, *i.e.*, in the range of hundreds of nanogram per lire to tens of microgram per lire [28-31]. Due to their potentially harmful effect on aquatic ecosystem and human health, development of a treatment process for effective removal of TrOCs has gained significant interest in the recent years.

Different physicochemical and biological treatment technologies have been investigated for the removal of TrOCs from water and wastewater over the last decade, showing promising results [32-35]. However, each treatment process has its own advantages and disadvantages. For instance, the use of physicochemical processes (*e.g.*, coagulation and adsorption) may lead to the production of toxic sludge, and the disposal of the toxic sludge can be problematic [24]. Similarly, high retention membrane separation processes such as nanofiltration (NF), forward osmosis (FO) and membrane distillation (MD) can effectively retain TrOC but without their mineralization into non-toxic compounds [36-38]. Despite being the environmentally friendly and potentially cost-effective techniques, biological treatment processes (*e.g.*, CAS process) are only effective for certain groups of TrOCs such as hydrophobic compounds and/or compounds with strong electron donating groups (EDGs) [39, 40]. For effective removal of TrOCs, high retention membrane separation processes such as nanofiltration (NF)/reverse osmosis [41, 42] and membrane distillation [38, 43, 44] have been combined with membrane bioreactors (MBR) as a post-treatment step, providing effective removal to produce TrOC-free effluent stream. To avoid an additional high retention membrane separation process, the high retention (HR)-MBRs have been developed, which can achieve TrOC retention by membrane and subsequent biodegradation in a single step for the production of high quality effluent suitable for water reuse applications [40].

HR-MBR combines the high retention membranes such as nanofiltration (NF), forward osmosis (FO) or membrane distillation (MD) with a CAS process. Available studies report that HR-MBR provides effective removal of a wide range of TrOCs [45, 46]. One of the underlying rationales for the development of HR-MBR was that the effective retention of pollutants within the bioreactor may facilitate biodegradation due to the prolonged contact time between the activated sludge and

TrOCs. Despite the effective TrOC retention achieved by the high retention membranes [45], degradation of TrOCs by the activated sludge within the bioreactor has not been reported to consistently improve [45, 46]. This is because the degradation of TrOCs by the activated sludge depends on their intrinsic biodegradability that is governed by their physicochemical properties such as chemical structure and hydrophobicity [47]. Poor degradation of resistant TrOCs HR-MBR leads to their accumulation within the bioreactor of HR-MBR. To improve the degradation of TrOCs in HR-MBR, other microbes with better TrOC degradation capacity than the conventional activated sludge can be introduced. In this context, white-rot fungi (WRF) and their ligninolytic extracellular enzymes [48] are worth-noting.

Depending on growth medium and culture conditions as well as on the type of WRF species/strains, WRF can secrete four different ligninolytic enzymes namely laccase, lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP). In addition, cytochrome P450 monooxygenases, a group of intracellular enzymes, have also been reported to play a vital role in the degradation of TrOCs *via* hydroxylation, dehalogenation and heteroatom oxygenation mechanisms [49-51]. Whole-cell WRF and their ligninolytic enzymes have been reported to efficiently remove a wide range of TrOCs such as pharmaceuticals (*e.g*., ibuprofen, ketoprofen and diclofenac), ingredients of personal care products (*e.g.*, triclosan and oxybenzone) and steroid hormones [52-55]. The capacity of WRF for TrOC removal has been commonly investigated under sterile conditions to avoid bacterial contamination. However, several studies have cast light on the aspect of bacterial contamination by operating bioreactors under non-sterile environment using either synthetic [55, 56] or real wastewater [57-62]. For example, Yang et al. [55] investigated the performance of whole-cell *Trametes versicolor* for the removal of bisphenol A and diclofenac in a membrane bioreactor under non-sterile conditions using a malt-based synthetic wastewater. They observed that the removal of diclofenac was reduced by 40-50% under non-sterile conditions as compared to its 99% removal achieved in sterile batch experiments. In that study, bacterial contamination was evident from microbial analysis. A few recent studies have investigated the removal of pharmaceuticals and endocrine disrupting compounds from municipal and hospital wastewater by whole-cell *Phanerochaete chrysosporium* or *Trametes versicolor* [57-63]. In all these studies, bacterial contamination restricted long term operation of the bioreactors as the overall removal of the TrOCs gradually reduced as compared to that obtained under sterile conditions.

Use of the harvested enzyme instead of a live whole-cell preparation allows decoupling of fungal growth and pollutant degradation steps, and this can be a suitable strategy to avoid bacterial contamination issues. Importantly, harvested enzymes can achieve TrOC degradation under mild conditions, while realizing higher rates and reaction specificity [64]. Degradation of TrOCs,

particularly by laccase has been extensively investigated in recent years [53, 65, 66]. Despite the promising performance of laccase for TrOC degradation, a few research gaps need to be addressed for improving the efficacy of enzymatic bioreactors. These research gaps are explained in section 1.2, and are addressed in Chapter 3-7 of this thesis (see Section 1.4)."

1.2. Knowledge gaps

Initial studies have assessed the performance of laccase-catalyzed TrOC degradation in batch enzymatic bioreactors due to the concern of enzyme washout in a continuous-flow system. In an attempt to prevent enzyme washout, an enzymatic membrane bioreactor (EMBR) was developed by coupling an ultrafiltration (UF) membrane to an enzymatic bioreactor [53, 67]. Membrane coupling to an enzymatic bioreactor offers several advantages over other alternatives such as: (i) more effective enzyme retention compared to packed bed reactors; (ii) avoid mass transfer limitation linked with enzyme immobilization; and (iii) easy to replenish enzyme for prolonged operations [64, 68]. Notably, during the operation of a UF-EMBR, adsorption of some hydrophobic TrOCs (*e.g.*, amitriptyline, oxybenzone and octocrylene) onto the enzyme gel layer over the membrane surface resulted in enhanced degradation of the adsorbed compounds [53]. In another study, removal of four non-phenolic TrOCs, namely atrazine, sulfamethoxazole, diclofenac and carbamazepine was improved by 15–25% following the addition of granular activated carbon (GAC) in a UF-EMBR. This was probably because simultaneous adsorption of laccase and TrOCs on GAC promoted the interaction of TrOCs with the active sites of laccase [69]. Results from these studies indicate the complementarity of simultaneous laccase and TrOC retention within an EMBR in contrast to only laccase retention by UF membranes utilized in the previously developed UF-EMBRs. However, the impact of simultaneous retention of both laccase and TrOCs by integrating an enzymatic bioreactor with a high retention membrane separation process (*e.g.*, NF and MD) has not been systematically assessed, and performance governing factors have not been elucidated.

Degradation of TrOCs in an enzymatic bioreactor can be improved by adding different natural and synthetic redox-mediators that are low molecular weight compounds capable of exchanging electrons between laccase and TrOCs [66, 70, 71]. Studied report that the addition of redox-mediators can extend the spectrum of efficiently degraded TrOCs [53, 72]. However, inhibition of laccase activity following the addition of redox-mediators has been observed. For instance, Hata et al. [73] observed 90% reduction in laccase activity within fist 8 h of incubation in the presence of 1-hydroxibenzotriazole (HBT). Rapid decline in laccase activity was also observed following the addition of HBT, syringaldehyde (SA) and 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfnoic acid (ABTS) separately [74]. Rate of laccase inactivation depends on the relative stability of the radicals generated by redox-mediators. Despite rapid inactivation of enzymes, redox-mediators

can compensate by enhancing the rate of reaction, eventually achieving rapid and enhanced removal of TrOCs. Hence, it is worthwhile to assess the effect of redox-mediator types and concentration on TrOC removal and laccase stability in a high retention (HR)-EMBR capable of retaining both laccase and TrOCs. The conventional UF-EMBRs requires continuous mediator dosing for achieving stable TrOC removal because redox-mediator pass through the UF membrane during filtration of bioreactor media. A high retention membrane processes integrated with an EMBR is expected to retain redox-mediators and may allow long-term operation of EMBR without mediator re-injection.

Advanced oxidation processes (AOPs) such as photolysis and persulfate oxidation produce highly reactive radicals (*e.g.*, hydroxyl and sulfate radicals) that can directly oxidize a wide range of TrOCs [75]. A combination of an AOP and laccase-catalyzed degradation process may act synergistically and could achieve effective TrOC degradation. Because an integrated AOP-laccase assisted membrane bioreactor system for TrOC removal has not been assessed previously, it will contribute to the body of knowledge on the fate of TrOCs in integrated treatment processes. It will also help to elucidate the role of a high retention membrane separation process during continuous treatment.

Laccase-catalyzed degradation of TrOCs, particularly in the presence of mediators, produces reactive radicals and transformation products that may increase the toxicity of the treated effluent [76, 77]. In addition, estrogenic activity is another important parameter to evaluate the safety of treated effluent for disposal and reuse [78, 79]. To predict the risk associated with the disposal of treated effluent, bioassays have been developed and reported for quantifying the toxicity and estrogenicity [80, 81]. However, studies on TrOC degradation do not always report the toxicity and estrogenic activity, particularly when treating a mixture of a broad spectrum of TrOCs at an environmentally relevant concentration.

Physicochemical properties of raw water collected from surface water and groundwater are diverse. Raw water matrix contains different dissolved organic (*e.g.*, humic substances) and inorganic (*e.g.*, heavy metals) impurities. A treatment process capable of effectively removing both heavy metals and TrOCs from wastewater should be critically assessed. A few lab-scale studies on the removal of TrOCs and heavy metals are available [84, 85], but the impact of impurities such as metal salts on pollutant retention and membrane fouling remains largely unexplored.

1.3. Research objectives

The primary objective of this thesis is to develop and assess a high retention (HR)- enzymatic membrane bioreactor (EMBR) system for achieving enhanced TrOC removal. The main idea was

to gain an in-depth understanding of removal mechanisms and the factors affecting the performance of HR-EMBR. The specific research objectives are as follows:

i. To elucidate the impact of simultaneous laccase and TrOC retention on degradation by comparing the performance of a nanofiltration (NF)-EMBR with a 'control' ultrafiltration (UF)-EMBR under identical operating conditions.

ii. To systematically assess the performance of two high retention membranes including membrane distillation (MD) and nanofiltration (NF) coupled to an EMBR separately for understanding the role of membrane in TrOC and heavy metal removal as well as for analyzing the hydraulic performance and stability of the developed treatment systems.

iii. To critically assess the impact of redox-mediator (both natural and synthetic) type, concentration and mixture on TrOC degradation, laccase stability and effluent toxicity in both NF- and MD-EMBRs.

iv. To elucidate the factors affecting the performance of an integrated laccase/persulfate assisted oxidation process on TrOC degradation, laccase stability, effluent toxicity and estrogenicity.

1.4. Thesis outline

This thesis is divided into eight chapters (**Figure 1.1**), and the research objectives outlined above in **Section 1.2** has been addressed in Chapters 3-7.

Chapter 1 describes the background, research gaps and research objectives of this study.

In *Chapters 2*, a comprehensive literature review of the occurrence of TrOCs in wastewater, freshwater bodies and seawater. In addition, status and evolution of current activated sludge-based biological processes such as MBR and HR-MBR are critically explained to understand the fate of TrOCs during wastewater treatment. The current knowledge related to the application of WRF and their ligninolytic enzymes (particularly laccase) for TrOC degradation is provided, and the performance governing factors along with research gaps are identified.

Chapter 3 is arguably the most critical chapter of this thesis because it serves as a proof of the concept – simultaneous retention of TrOC and laccase within enzymatic bioreactor facilitate degradation. This is achieved by comparing the performance of a NF-EMBR and a 'control' UF-EMBR under identical operating conditions.

In *Chapter 4*, another configuration of HR-EMBR (*i.e.*, MD-EMBR) is assessed for enhanced TrOC degradation, and the impact of laccase source on the extent of degradation are elucidated. The purpose of this chapter is to demonstrate that other high retention membrane separation

processes, in addition to NF membrane, can be integrated with an enzymatic bioreactor for achieving improved TrOC degradation.

In *Chapter 5*, long-term performance of MD-EMBR for the removal of 30 TrOCs having diverse physicochemical properties (*e.g.,* EDGs/EWGs, hydrophobicity and phenolic/non-phenolic moieties) is examined. The effect of dosing redox-mediators, separately and as a mixture, on TrOC degradation, laccase stability and effluent toxicity is elucidated.

In *Chapter 6*, a novel integrated laccase/persulfate oxidation process is examined for the first time. The effect of persulfate concentration and incubation time on TrOC degradation, toxicity and estrogenicity is elucidated in both batch bioreactor and continuous-flow NF-EMBR.

In *Chapter 7*, simultaneous removal of both TrOCs and the selected heavy metals by MD process is assessed. The effect of metal ions and organic impurities on membrane retention and fouling is systematically studied. The MD process was selected for simultaneous TrOC and heavy metal removal because the literature suggests better performance of MD process as compared to the NF membrane.

Finally, the *Chapter 8* summarizes the key findings of this study and outlines recommendations for the future research.

In this thesis, *Chapters 3-7* are structured as a scientific publication, with their own introduction, materials and methods, conclusion and reference sections; and its own supplementary information placed at the end of the thesis. However, where suitable, reference to a section in a previous chapter is made to avoid repetition in materials and methods section.

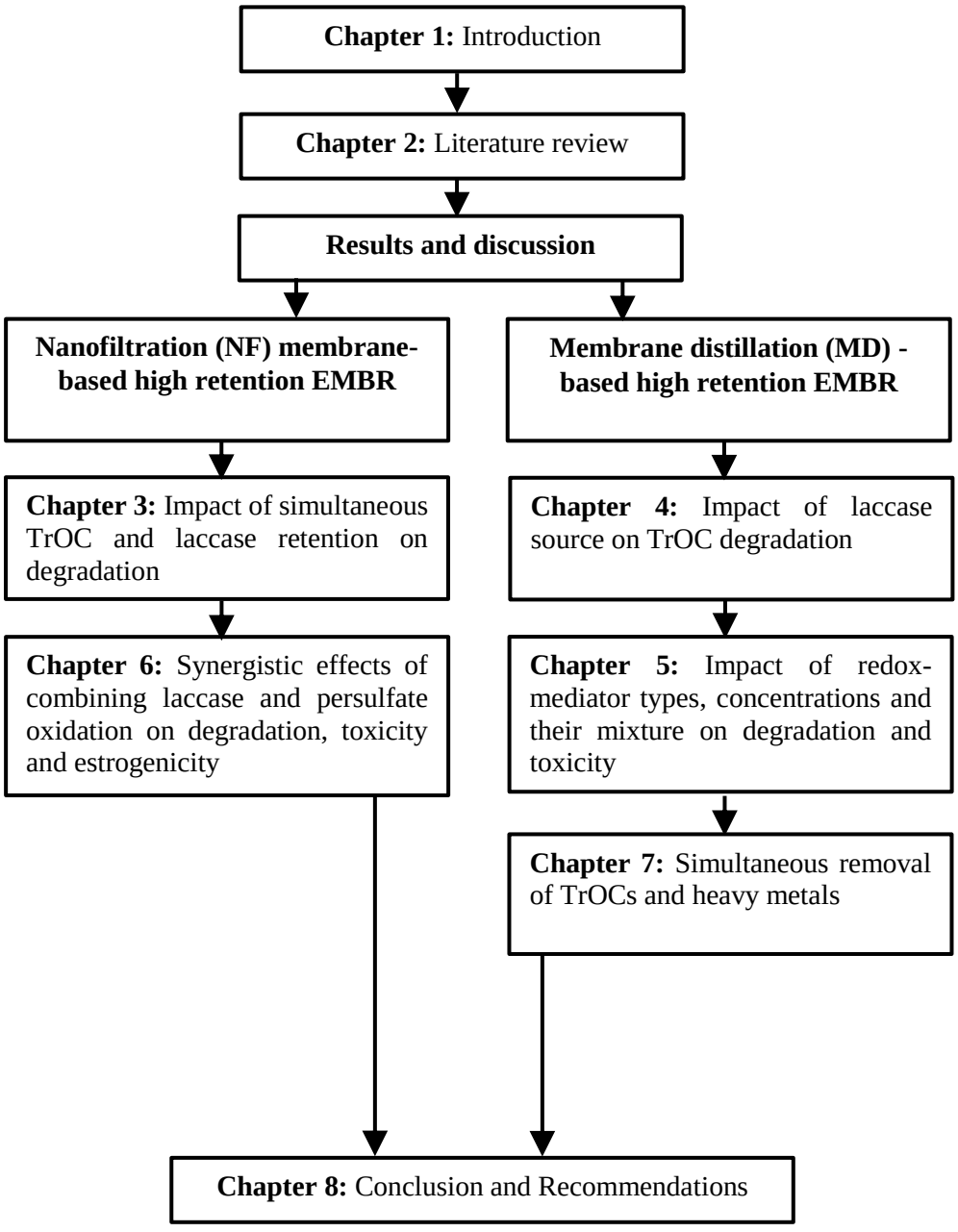

Figure 1.1. A schematic representation of thesis structure and outline

1.5. Contribution to knowledge

Overall aim of this thesis is to develop an integrated process for improving the degradation of TrOCs, which is a significant addition in the existing literature. The integrated treatment process that is developed and studied for the first time in this thesis could be applicable to other studies dealing with bioremediation of hazardous pollutants such as phenols. Since a high retention

enzymatic membrane bioreactor (HR-EMBR) has not been studied previously, the findings of this thesis contribute significantly by identifying the performance governing factors as well as by providing an in-depth understanding of the fate of TrOCs during laccase catalyzed degradation in EMBR.

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Chapter 2: Literature Review

This chapter is based on the following publications:

Journal articles:

Asif, M.B., Hai, F.I., Singh, L., Price, W.E., Nghiem, L.D. 2017. Degradation of pharmaceuticals and personal care products by white-rot fungi—A critical review. *Current Pollution Reports, 3(2), 88-103.*

Asif, M.B., Ansari, A.J., Chen, S.-S., Nghiem, L.D., Price, W.E., Hai, F.I. 2018. Understanding the mechanisms of trace organic contaminant removal by high retention membrane bioreactors: a critical review. Environmental Science and Pollution Research, 1-16. DOI: https://doi.org/10.1007/s11356-018-3256-8

Book chapters:

Asif, M.B., Hai, F.I. 2019. Degradation of pharmaceutically active compounds by white-rot fungi and their ligninolytic enzymes. in: *Pharmaceutical Biocatalysis*, (Eds.) P. Grunwald, Pan Stanford Publishing, Singapore. (*in press*)

Asif, M.B., Hai, F.I., Jegatheesan, V., Price, W.E., Nghiem, L.D., Yamamoto, K. 2019. Applications of Membrane Bioreactors in Biotechnology Processes. in: *Current Trends and Future Developments on (Bio-) Membranes: Membrane processes in the pharmaceutical and biotechnological field*, (Eds.) A. Basile, C. Charcosset, Elsevier. Amsterdam, Netherlands, pp. 223-257. (ISBN: 9780128136065)

2.1. Introduction

In this section, rationale for carrying out this research is outlined, and the arrangement of the literature review is explained. Due to their ineffective removal by conventional wastewater treatment plants (WWTPs), widespread occurrence of trace organic contaminants (TrOCs) has been reported in freshwater bodies (*See* **Section 2.2 and 2.3**). For their effective removal, a high retention membrane bioreactor (HR-MBR) was developed, which combines the high retention membranes such as nanofiltration (NF) or membrane distillation (MD) with an activated sludge. Activated sludge-based HR-MBR provides effective removal of a wide range of trace organic contaminants (TrOCs), *via* membrane retention and biodegradation, and can produce high quality TrOC-free effluent stream for safe disposal and reuse. One of the underlying rationales for the development of activated sludge- based HR-MBR was that the effective retention of pollutants within the bioreactor may facilitate biodegradation due to the prolonged contact time between the activated sludge and TrOCs. Despite the effective TrOC retention by the high retention membranes, degradation of TrOCs by activated sludge within the bioreactor has not been reported to consistently improve (*see* **Section 2.3.3.4**). This is because the degradation of TrOCs by the activated sludge depends on their intrinsic biodegradability. To improve the degradation of TrOCs in HR-MBR, other microbes with better TrOC degradation capacity than conventional activated sludge can be introduced. In this context, white-rot fungi (WRF) and their extracellular ligninolytic enzymes (such as laccase) are worth-noting. They can achieve effective degradation of TrOCs that are resistant to an activated sludge-based treatment process (*see* **Section 2.4.2**). However, bacterial contamination may hamper the growth and enzymatic activity of whole-cell WRF in any reactor configuration. Use of the harvested enzymes, particularly laccase instead of a whole-cell preparation allows decoupling of fungal growth and pollutant degradation steps, and this can be a suitable strategy to avoid bacterial contamination issues. Initially, only batch enzymatic bioreactors were assessed for the degradation of TrOCs to prevent enzyme washout with the treated effluent (*see* **Section 2.4.2)**. This led to the development of a laccase-based EMBR that couples an enzymatic bioreactor with an ultrafiltration (UF) membrane having a suitable molecular cutoff for effective enzyme retention (*see* **Section 2.4.4)**. UF membranes in practice cannot effectively retain TrOCs. Thus, TrOCs that are not readily degraded by laccase can still pass through the UF membrane, consequently requiring an additional post-treatment process (*e.g.*, high retention membrane separation processes) for their effective removal.

It is important to note that the formation of an enzyme gel-layer on the surface of the membrane in previously developed UF-EMBR effectively adsorbed a few significantly hydrophobic TrOCs (log D >3). This resulted in improved degradation of the adsorbed TrOC following their retention by the enzyme gel-layer (*see* **Section 2.4.4)**. Hence, it was envisaged that the use of high retention

membranes, which will retain both laccase and TrOCs, can facilitate the degradation of resistant TrOCs in HR-EMBR. Importantly, HR-EMBR will produce high quality TrOC-free effluent without an additional post-treatment process. In light of the discussion above, literature review is structured accordingly in four different sections. In **Section 2.2**, recent occurrences and fate of TrOCs in surface water, groundwater and seawater are systematically presented and discussed. In addition, the factors influencing the occurrence of TrOCs in different environmental systems are elucidated. **Section 2.3** discusses the performance of biological processes (such as conventional- and HR-MBRs), and critically analyses the factors governing the removal of TrOCs. **Section 2.3** is critical to understand the fate of TrOCs during biological treatment, and it facilitates in identifying the research gaps that led to the development of enzymatic-MBRs. **In Section 2.4**, performance of WRF and their ligninolytic enzymes for TrOC removal is critically evaluated. TrOC removal by enzymatic membrane bioreactor (EMBR) is particularly discussed, and the role of membrane in removal is elucidated. In addition to exploring the efficiency of enzymatic degradation, this thesis explores the combined application of enzymatic and emerging advanced oxidation processes (AOPs). In line with this, performance of emerging AOPs such as persulfate oxidation process is reviewed and presented in **Section 2.5.**

2.2. Trace organic contaminants (TrOCs)

A broad spectrum of organic compounds has become an integral part of our daily life. These compounds are used in immense quantities for a variety of purposes including industrial processes, food production and preservation as well as for the healthcare of human and animals [1-3]. Occurrence of these compounds in environmental systems has become a topic of growing interest over the last decade due to their potential detrimental impacts on both aquatic life and human health [4, 5]. Among these compounds, TrOCs, also known as emerging organic pollutants, are of particular interest due to their widespread occurrence in wastewater as well as in different environmental systems such as groundwater, surface water and seawater. TrOCs from both natural and anthropogenic sources can be divided into different categories including pharmaceuticals, personal care products, pesticides, industrial chemicals, steroid hormones and food preservatives [6, 7]. Concentration of TrOCs in environmental system could be very low (*i.e.*, in the range of a few µg/L to ng/L). This not only affects the analytical procedures associated with their detection [8], but may also influence the efficacy of water and wastewater treatment processes.

Conventional wastewater treatment plants (WWTPs) have not been designed for effective TrOC removal [9]. Following the discharge of the treated effluent, TrOCs may induce ecotoxicity and endocrine disrupting effects in an aquatic ecosystem [10-14]. Although occurrence, fate and impacts of TrOCs have been extensively studied in last 15 years, their environmental significance is still not fully understood. Different organizations including the World Health Organization

(WHO), the European Union (EU), the International Program of Chemical Safety (IPCS) or the North American Environmental Protection Agency (EPA) have realized these issues and are developing legal frameworks to protect environmental systems, particularly freshwater sources [15]. A wealth of studies have been published in the last decade on the occurrence of TrOCs in surface water [8, 16, 17] and groundwater [18], as well as their fate in biological and physicochemical treatment processes [6, 9, 16]. In this section, recent occurrences and fate of TrOCs in surface water, groundwater and seawater are systematically presented and discussed. In addition, the factors influencing the occurrence of TrOCs in different environmental systems are elucidated.

2.2.1. Occurrence of TrOCs in environmental systems

With ever growing population, pharmaceutical production for human use has increased many folds in recent years [19, 20]. According to one estimate, thousands of new pharmaceuticals are being invented every year [21]. Per capita consumption of pharmaceuticals in developing countries varies from 50-150 g/year. However, worldwide average of pharmaceutical consumption is on the lower side (15 g/capita.year) due to their less consumption in developed countries [22]. Upon consumption, pharmaceuticals and animal medicines are excreted into the wastewater, making it the most significant source of TrOC in environmental systems [23]. Similarly, other sources of a broad spectrum of TrOCs and their intermediates in different environmental systems include but are not limited to hospital wastewater, industrial effluents, aquaculture and livestock activities, solid waste dumping and agricultural runoff [18, 24, 25]. Notably, domestic use of pharmaceuticals and personal care products remains the largest contributor of TrOC contamination in wastewater and subsequently in environmental systems. Sources and pathways of TrOCs in the surface water, groundwater and seawater are shown in **Figure 2.1**. Domestic, industrial and hospital wastewater in addition to the effluent from combined sewage treatment plant are the examples of point source, and TrOC loading from these sources can be measured and monitored. On the other hand, diffuse sources such as urban/agriculture/livestock runoff, biosolids, artificial recharge and leachate originating from a large geographical area cannot be quantified accurately (**Figure 2.1**). Uncertainty of TrOC loading in the freshwater and seawater sources originating from diffuse sources is the major challenge to measure, assess, control and monitor their detrimental impacts [1, 18, 26].

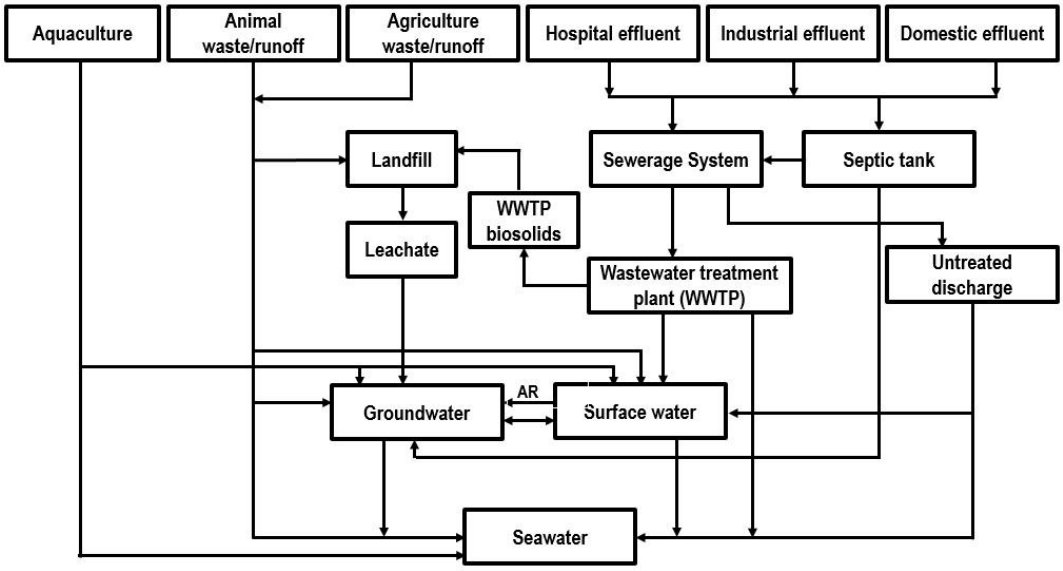

Figure 2.1. Sources and pathways of TrOC contamination in freshwater and seawater. Data source: [1, 18, 26]. "AR": artificial recharge

2.2.2. Wastewater

Reported concentrations of TrOCs in the influent and effluent of WWTPs summarized in **Table 2.1** indicate significant variations among the selected compounds. Variations in the influent concentration of TrOCs can be attributed to several factors such as the production rate, annual sales, excretion rate, agricultural practices and average daily water consumption per person. On the other hand, effluent concentration of TrOCs may vary, depending on the relevant environmental regulations and effectiveness of WWTPs. Since most of the operational WWTP were not designed for effective TrOC removal, poor TrOC removal is not entirely unexpected [27, 28]. According to **Table 2.1**, influent concentration of TrOCs varies between 1 and 10 µg/L, while the concentration of some pharmaceuticals such as atenolol, caffeine, naproxen and diclofenac, one industrial chemical (DEHP), one pesticide (triclosan) and one surfactant (nonylphenol) is relatively high (up to 1 mg/L) in raw wastewater. TrOCs with high concentration in raw wastewater are mostly pharmaceuticals, probably because of their elevated production amounts and use/consumption in our daily life. It is important to note that the prescription may not be required for a few pharmaceuticals such as ibuprofen and caffeine. Thus, their occurrence at high concentration can be attributed to the ease of accessibility.

Concentration of steroid hormones has been reported to be generally low (<1 µg/L) in raw wastewater (**Table 2.1**). Average excretion rate for steroid hormones ranges between 0.4 and 81 µg/capita.day (**Table 2.1**). Effluent concentration of most TrOCs in WWTP varies from 1 to 10

µg/L and governed by the efficacy of WWTPs. However, a few TrOCs such as acetaminophen, caffeine, DEHP, atenolol and triclosan are discharge into water bodies at high concentrations (up to 40 µg/L) due to their abundance in wastewater and/or their persistence to conventional wastewater treatment process. Steroid hormones are generally well removed in conventional WWTPs, but their very low concentration (up to 1 ng/L) is enough to induce estrogenic effects in aquatic life [29].

In general, production of TrOCs and their use/consumption pattern in a country govern the level TrOC contamination in wastewater. Many studies have correlated the concentration of pharmaceuticals in wastewater with their production as well as with the population of a country. For instance, Kasprzyk-Hordern et al. [23] and Choi et al. [30] studied the occurrence of pharmaceuticals such as acetaminophen, carbamazepine, sulfamethoxazole, codeine in the wastewater of UK and Korea, respectively. They observed that the concentration of pharmaceuticals in wastewater followed the production rate of these TrOCs in their respective countries. However, a few studies reported that the concentration of some TrOC in wastewater did not correlate well with population and their production rates [31, 32], possibly because TrOCs can find their way into wastewater steams from other sources such as storm water runoff a (**Figure 2.1**). Since pharmaceuticals require oral ingestion, their occurrence in domestic wastewater also depends on their excretion through urine and feces. Excretion rate of ibuprofen, carbamazepine, naproxen, clofibric acid and gemfibrozil is generally low (1-10%). On the other hand, the highest excretion rate (>70%) has been reported for paracetamol and atenolol (**Table 2.1**). However, it is important to note that high or low excretion rate does not necessarily mean high or low concentration of these TrOCs in wastewater. In addition to the ambient environmental conditions, outbreak of a certain disease in a community could also influence the concentration of pharmaceuticals and personal care products in raw wastewater. Moreover, concentration of pesticides in wastewater could be influenced by seasonal variations [16, 33]. Similarly, rainfall effects the flow pattern of wastewater in combined sewerage system, resulting in the change of wastewater composition containing TrOCs. For instance, Kasprzyk-Hordern et al. [23] observed an increase (up to 2 folds) in the concentration of pharmaceuticals in domestic wastewater during dry weather conditions, as compared to rainy season.

Table 2.1. Occurrence of a wide range of TrOCs in wastewater. The range of influent concentration, effluent concentration and removal efficiency of TrOCs is presented. Excretion rates of pharmaceuticals and steroid hormones are also given. Data is extracted from [16, 17, 29, 34].

Category	Compounds	Excretion rate	Concentration (µg/L)		Removal efficiency
		(%)	Influent	Effluent	(%)
Pharmaceuticals					
β-blocker	Atenolol	50-95	0.14-35.1	0.33-7.60	0-80
	Metoprolol	5-30	0.019-1.48	0.003-0.30	3-56
	Propranolol	2.5-10	0-0.5	0.30-0.71	40-45
	Sotalol	0-17.5	0.320-0.711	0.21-0.24	25-35
Analgesic and anti-inflammatory	Acetaminophen	≤5	5.64-44	21-70	0-0.03
	Diclofenac	0.5-39	0.001-90.5	<0.001-0.69	0-81
	Ibuprofen	5-10	0.004-590	0-55	70-100
	Ketoprofen	-	0.004-9.01	<0.001-3.42	10-100
	Mefenamic acid	-	0.015-1.15	0.005-0.40	0-75
	Naproxen	≤0.5	<0.01-50.7	0.002-5.1	43-99
	Salicylic acid		0.58-65.4	0-0.54	90-100
Anticonvulsant	Carbamazepine	3-5	0.035-3.8	<0.01-4.50	0-65
Lipid regulator	Bezafibrate	40-69	0.05-1.39	0.03-0.67	10-70
	Clofibric acid	≤6	0-0.74	ND-0.33	0-93
	Gemfibrozil	≤1	0.10-17.1	<0.01-5.24	0-90
Antibiotic	Erythromycin	5-25	0.14-10.0	0.02-2.84	0-82.
	Sulfamethoxazole	20	<0.01-0.98	<0.01-1	15-90
	Trimethoprim	≤39	0.06-6.80	<0.01-3.05	0-80
Nervous stimulant	Caffeine	1-5	0.22-209	0-43.50	50->99
Personal care products					
Musk fragrance	Galaxolide	-	0.03-25	0.06-2.77	85-88
	Tonalide	-	0.05-1.93	0.05-0.32	80-85
Disinfectant	Triclosan	-	0.03-23.9	0.01-6.88	71->99
Insect repellant	DEET	-	2.56-3.19	0.61-15.8	66-80
UV filter	Benzophenone	-	0.079-0.90	0.079-0.23	64-98
Steroid hormone					
	Estrone (E1)	19 [a]	0.01-0.17	0.001-0.08	75-90
	Estradiol (E2)	7.7 [a]	0.002-0.05	0.001-0.007	93-100
	17α-Ethynylestradiol (EE2)	0.41 [a]	0.001-0.003	0.001-0.002	45-100
	Estriol (E3)	81 [a]	0.125-0.80	0	100

Category	Compounds	Excretion rate	Concentration (µg/L)		Removal efficiency
Surfactants					
	Nonylphenol	-	0.03-101.6	0.03-7.8	25-99
	Octylphenol	-	0.2-8.7	0.004-1.3	0-95
Industrial Chemicals					
Plasticizers	Bisphenol A	-	0.013-2.14	0.03-1.10	65-99
	DBP	-	0-11.8	0-4.13	75-80
	DEHP	-	0.003-70.0	0.0001-54.0	25-97
	DMP	-	0-6.49	0-1.52	85-95
Fire retardant	TCEP	-	0.06-0.50	0.06-2.40	0
	TCPP	-	0.18-40.	10-21	0
Pesticides					
Herbicide	Atrazine	-	0.02-28	0.004-0.73	0-25
	Diuron	-	0.03-1.96	0.002-2.53	25-70
	Diazinon	-	0-0.684	0.0007-4.16	0
Fungicide	Clotrimazole	-	0.012-0.08	ND-0.005	85-95
Insecticide	Tebuconazole	-	0-1.89	0.0005-0.69	0-60

"–": not available; ND: not detected
[a] µg/capita.day

2.2.3. Surface water

Compared to other sources (**Figure 2.1**), major source of TrOCs in surface water has been reported to be the discharge of the treated effluent from WWTPs [17, 35]. After the discharge of TrOCs into freshwater bodies, different natural attenuation processes such as photolysis, aerobic biodegradation, sorption onto sediments and dilution play an important role. However, in-stream attenuation rate varies for each process, and is dependent on the physicochemical characteristics of TrOCs and local environmental conditions. For instance, Kunkel and Radke [36] observed different attenuation rates for 10 pharmaceuticals in a river, and the physicochemical properties were credited for these variations. Similarly, while investigating the relationship between natural attenuation rates and physicochemical properties of 225 TrOCs [37], high attenuation rates were obtained for: (i) compound having medium to low volatility ($-4<\log K_{aw}<-2$); and (ii) hydrophilic compounds ($0<K_{ow}<4.5$). By contrast, Acuñaet al. [38] did not observe any meaningful correlation between attenuation rate and physicochemical properties of TrOCs. Hence, more studies are needed to understand the factor affecting the natural attenuation of TrOCs in environmental systems.

Photolysis is an important natural attenuation process for TrOCs in surface water bodies. However, Kunkel and Radke [36] found that some TrOCs such as bezafibrate, metoprolol and naproxen

could not be attenuated *via* photolysis. Despite the efficacy and rapidness of photolysis process, resultant transformation byproducts could be toxic and resistant to further photolysis or biodegradation. For instance, Donner et al. [39] showed that the transformation byproducts formed during photolysis were more toxic than their parent compound (*i.e.,* carbamazepine). Water dilution and sorption onto sediments also contribute in attenuating a wide range of TrOCs. Significance of water dilution can be realized from the fact that high concentration of TrOCs in surface water bodies has been reported during dry weather [40, 41]. Concentration of TrOCs in surface water was less in samples collected during summer season than those collected during winter season [42]. Enhanced biodegradation rate due to high temperature in summer and/or elevated wetter summer season could be another reason of relatively lower TrOC concentration in summer than winter [42]. Rainfall, in some cases, can also contribute as a source of TrOCs in surface water bodies. This is because they can leach from municipal solid waste dumping sites during the rainfall and ends up in either combined sewerage system or surface water bodies [43, 44].

Contamination of surface water bodies with pesticides may depend on the characteristic of receiving water body such as: flows rate and depth; distance from land; soil characteristics; and crop type [45]. While studying the pathways of TrOCs for Swist river basin (Germany), Christoffels et al. [44] detected the presence of pharmaceuticals in combined sewer overflows, and they also observed the presence of fungicides and insecticides in the runoff originating from an orchard, thus, highlighting the significance of diffuse sources for TrOCs. **Figure 2.2** illustrates the variations in the concentration (ng/L) of each class of TrOCs from recent studies. Pharmaceuticals are the most commonly detected class of TrOCs followed by industrial chemicals. Nonsteroidal anti-inflammatory drugs (NSAIDs) such as acetaminophen, diclofenac and ibuprofen are the most frequently detected subclass of pharmaceuticals in surface water.

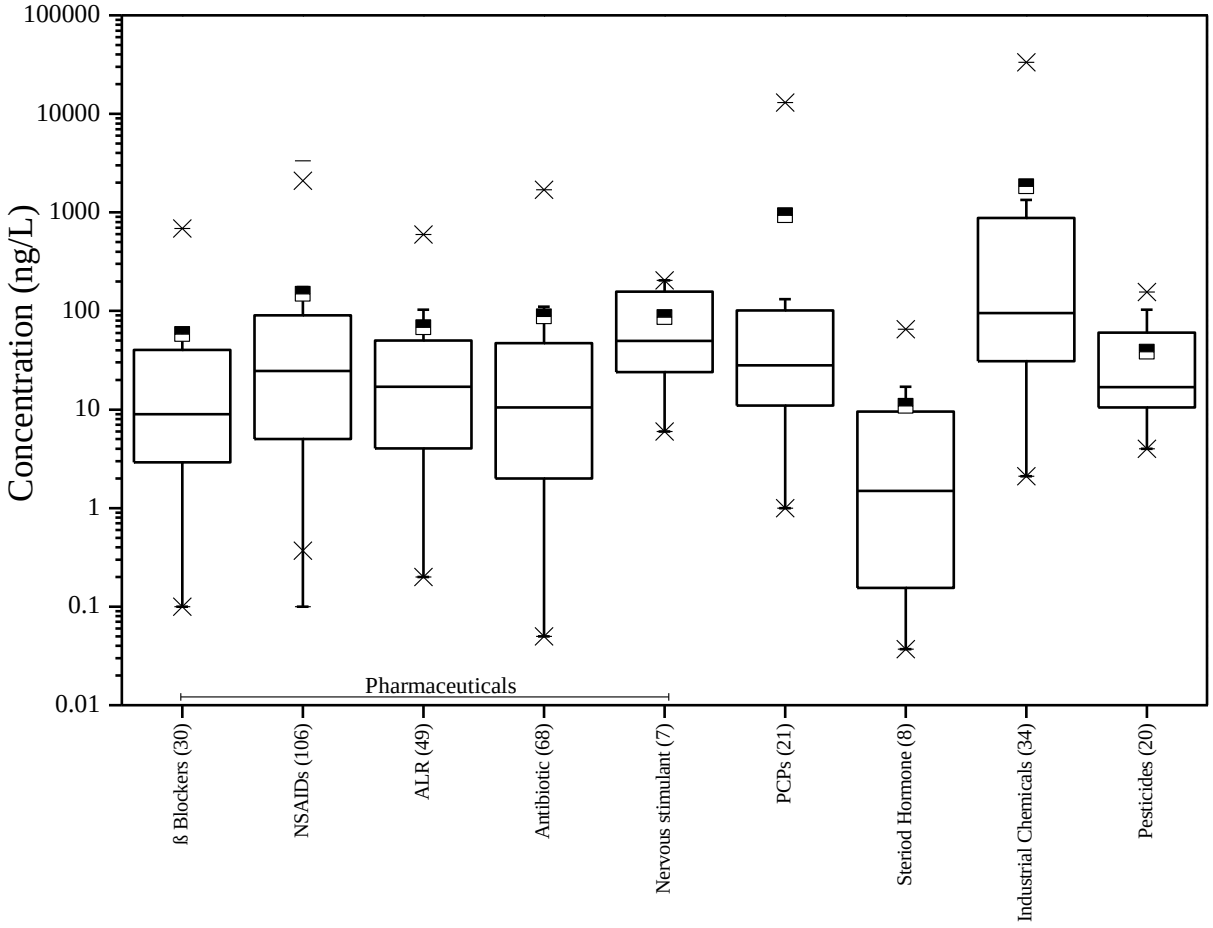

Figure 2.2. Variations in the concentration (ng/L) of TrOCs in surface water bodies. Box plots represents interquartile range, median (horizontal line), min and max (whiskers), and average (black and white square box). Number of data points for each class/subclass is given in brackets on the x-axis. NSAIDs: Nonsteroidal anti-inflammatory drug; ALR: Anticonvulsant and Lipid regulator; and PCPs: Personal care products. Data is extracted from [46-54].

Average concentration of pharmaceuticals varies from 75 to 140 ng/L (**Figure 2.2**) and the highest average concentration (140 ng/L) is observed for NSAIDs possibly due to their abundance in domestic wastewater (**Table 2.1**). On the other hand, average concentration of industrial chemicals (1150 ng/L) and personal care products (410 ng/L) in surface water is alarmingly high. Concentration of steroid hormones ranges from 1 to 10 ng/L, and is high enough to induce estrogenic effects [50].

2.2.4. Groundwater

Groundwater is the major source of freshwater for domestic and industrial use in many countries. It constitutes approximately 30% of total freshwater resources in this world. Since 70% of freshwater resources are frozen, groundwater represents 97% of freshwater available for human

use [55]. It also suggests that groundwater is key for sustainable water supplies in the world. Despite the high attenuation of some TrOCs in soil strata, some studies confirmed the presence of TrOCs in groundwater, making it an issue of significant concern [18, 56-58]. Currently, the occurrence of TrOCs in groundwater has been well documented only for Europe and America [56, 58-61]. Concentration of TrOCs may not be as high as in surface water bodies but still more emphasis is needed to characterize them better in other parts of the world.

TrOCs can contaminate groundwater from variety of sources such as wastewater, contaminated surface water, landfill leachate, artificial recharge, percolation of agriculture/storm water runoff and leakages from sewers and septic tanks (**Figure 2.1**). Concentration of TrOCs from septic tank/sewer leakage and landfill leachate generally varies from 10 to 10^4 and 10 to 10^3 ng/L, respectively, making them the major sources of groundwater contamination. Application of pesticides in agriculture lands may contaminate groundwater because they can percolate down with irrigation water [62]. After the discharge of WWTP effluent containing TrOCs in surface water bodies, TrOCs can also find their way into groundwater due to lateral and vertical hydraulic exchange [63]. For instance, Gasser et al. [64] reported the transfer of TrOCs from surface water to groundwater by using carbamazepine as a marker. Artificial recharge (AR) using surface and reclaimed water is another source of TrOCs in groundwater, especially when the reclaimed water has poor quality and residence time in soil is short. Common AR processes include but are not limited to well injection and river bank filtration [65]. TrOCs during the river bank filtration of WWTP effluent could find their way into shallow groundwater aquifer, and the concentrations of a few compounds such as carbamazepine and sulfamethoxazole were reported to be above 20 ng/L in groundwater along the bank of the stream [66].

TrOCs pass through subsurface and unsaturated zone before reaching the groundwater. During the transfer of TrOCs in soil, natural attenuation helps to reduce their concentration [67]. Attenuation of TrOCs in underground environment is possible *via*: (i) adsorption; and (ii) biodegradation. Physicochemical properties of TrOCs such as hydrophobicity, molecular weight and chemical structure governs their adsorption on soil strata. TrOCs with octanol–water partition coefficient (K_{OW}) of less than 1.5 tend to be highly mobile and are more likely to be detected in groundwater [68]. For instance, sulfamethoxazole (log K_{OW}=0.9) and caffeine (log K_{OW}=-0.07) have been commonly detected in groundwater due to their poor adsorption on soil [18]. A few TrOCs such as triclosan (log K_{OW}= 4.76) are only adsorbed by both silt loam and sandy loam soil, while a few TrOCs such as caffeine (log K_{OW}=-0.07) are only adsorbed by sandy loam soil. These results suggest that caffeine is more likely to reach groundwater than triclosan. Teijón et al. [69] found that low sorption potential of naproxen on sandy loam material with low organic content resulted in its ubiquitous occurrence in sandy loam aquifer. It also suggests that properties of TrOCs and

soil characteristics both govern the mobility of compounds in soil. Occurrence of TrOCs such as pharmaceuticals and personal care products in the soil of different countries along with their physicochemical properties is presented in **Table 2.2**. Concentration of a few TrOCs such as triclosan and ibuprofen is up to 35.5 µg/Kg due to high sorption capacity (log K_{ow} > 3). Despite having poor sorption coefficient (log K_{ow} > -0.9), concentration of oxytetracycline was 9.6 µg/Kg in the soils of China. It validates that other factors such as soil properties may also influence the occurrence of TrOCs in soil. Moreover, oxytetracycline, ibuprofen and triclosan in soil exceeds the predicted no effect concentration (PNEC) of these TrOCs in the soil of China and Mexico. In Europe, TrOCs with a log K_{ow} value of greater than 4 are considered an environmental risk [70].

It is believed that degradation of TrOCs is faster in aerobic conditions than anaerobic conditions, probably due to the difference in aerobic and anaerobic microbial communities [71]. Degradation of TrOCs in groundwater is generally poor and/or incomplete due to the unavailability of diverse microbial species in groundwater. However, while investigating the degradation of 27 pharmaceuticals under oxic and anoxic conditions, Burke et al. [72] reported effective degradation of some TrOCs such as propyphenazone, doxycycline and phenazone only in oxic conditions. On the other hand, some TrOCs including clindamycin, roxithromycin and clarithromycin were only degraded in anoxic conditions. Increase in temperature during summer season could facilitate the removal of a few TrOCs such as iopromide, metoprolol, and diclofenac in the hyporheic zone [73]. These observations presented here suggests that environmental parameters and redox conditions influence the degradation of TrOCs.

Table 2.2. Occurrence (µg/Kg) of some TrOCs in the soil of different countries. Data is extracted from [18, 74-79]

Compounds	Class/Use	Concentration (µg/Kg)					Log K_{ow}	PNEC (µg/Kg)
		USA[a]	China[b]	Mexico[c]	Malaysia[d]	India[e]		
Carbamazepine	Anticonvulsant	ND-1.4	0.02-0.06	0.1-16.4	-	-	2.45	48.6
Trimethoprim	Antibiotic	ND-0.64	0.64-2.15	-	3.1-60.1	-	-	-
Ibuprofen	NSAID	-	1.51-5.03	ND-0.3	-	-	3.97	3
Diclofenac	NSAID	-	0.35-1.16	<0.1	-	-	4.51	735
Sulfadiazine	Antibiotic	-	1.15-16	ND	-	-	0.28	-
Triclosan	Personal care product	ND	n.d-16.7	0.4-35.5	-	-	4.8	3.74
Oxytetracycline	Antibiotic	-	ND-9.6	-	-	-	-0.9	0.3
Norfloxacin	Antibiotic	-	61.9	-	-	0.011	1.03	47-12611
Salicylic acid	NSAID	-	4.5	-	-	-	2.26	2079
Ciprofloxacin	Antibiotic	-		-	-	0.014	3.1	248915
Ofloxacin	Antibiotic	-		-	-	0.019	-0.39	569
Naproxen	NSAID	-	-	0.20-2.40	-	-	3.18	2357

ND: not detected; NSAID: Nonsteroidal anti-inflammatory drug; K_{ow}: Octanol-water portioning coefficient; PNEC: Predicted no-effect concentration; and "–": not reported

Box and whisker plot (**Figure 2.3**) for the five classes of TrOC shows that pharmaceuticals are most commonly detected in groundwater, followed by the ingredients of personal care products. Average concentration of pharmaceuticals ranged from 100 to 1000 ng/L. Pharmaceuticals are of particular interest because of their widespread occurrence in wastewater and surface water (s*ee* **Table 2.1 and Figure 2.2**). Concentration of pesticides and personal care products is on the higher side (above 100 ng/L) in groundwater (**Figure 2.3**) as compared compared to surface water. This difference is because some studies reported the occurrence of TrOCs in the vicinity of a landfill site and septic tank [56, 80]. Concentration of steroid hormones is generally low (less than 10 ng/L) except for one study that reported a 17α-ethynylestradiol concentration of 1500 ng/L [32].

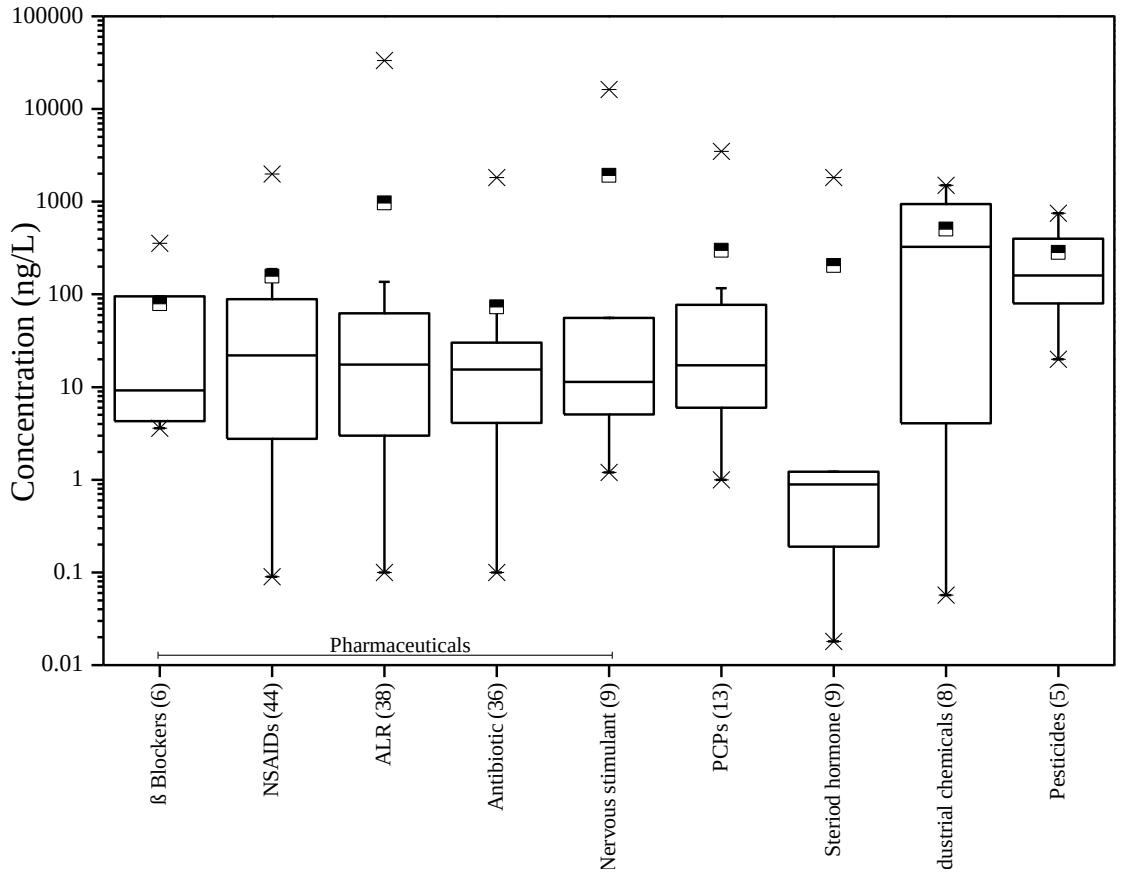

Figure 2.3. Variations in the concentration (ng/L) of TrOCs in groundwater. Box plots represents interquartile range, median (horizontal line), min and max (whiskers), and average (black and white square box). Number of data points for each class/subclass is given in brackets on the x-axis. NSAIDs: Nonsteroidal anti-inflammatory drug; ALR: Anticonvulsant and Lipid regulator; and PCPs: Personal care products. Data is extracted from [56, 57, 81-87].

2.2.5. Seawater

According to one estimate, one fifth of the world population lives in coastal areas. 21 out of 33 megacities of the world such as Mumbai (India), Guangzhou (China) and New York (USA) are situated in coastal areas [88]. Corcoran [89] estimated that above 80% untreated wastewater in coastal areas is discharged in to oceans without any treatment. Septic tanks are used for

wastewater collection in many Asian countries, and wastewater is subsequently released to coastal waters without any treatment [90]. A few cities such as Los Angeles discharge their treated effluent into oceans *via* marine outfalls [91, 92]. Following disposal in freshwaters, treated effluent also ends up in oceans, thereby putting an additional load of TrOC in seawater. According to one estimate, 150 tons of pharmaceuticals are received by the ocean *via* Yangtze River in China [93]. Ships, cruise liners and boats discharge their treated effluent in oceans [94]. Moreover, leachate from the landfill sites in coastal areas could also contaminate oceans. For instance, Rodríguez-Navas et al. [95] reported that the leachate from a landfill site situated in Mallorca Island (Spain) is adding 27 µg/L of pharmaceuticals in ocean on daily basis.

Aquaculture is one of the largest industry of the world, and more than 500 million people are associated with this industry [96]. A range of pharmaceuticals are used in aquaculture activities to prevent the outbreak of diseases. According to one estimate, more than 75% of the pharmaceuticals used during aquaculture find their way into oceans *via* food pellets and excretion [97]. Like surface water and groundwater, other sources of TrOCs in oceans include surface runoff, agriculture runoff and leaching from domestic/hospital waste [98, 99].

Unlike freshwater, occurrence and fate of TrOCs in oceans has been investigated only recently as >70% of the studies dealing with this topic are published in last ten years. However, more investigations are required for many parts of the world, especially South America, Asia and Africa. Interestingly, there is only one study reporting the occurrence of TrOCs in India, the 2nd largest population of the world [100]. Pharmaceuticals have been the most commonly reported class of TrOCs, while data availability is very limited for other classes such as personal care products, industrial chemical and pesticides. Scope of the studies included in this section varies significantly. Some studies deal with a particular class of TrOCs [101], while others focus on the development of detection methods and validation [102]. Occurrence of TrOCs in oceans is presented in **Figure 2.4**, which shows that pharmaceuticals have been commonly detected. Among pharmaceuticals, NSAIDs are ubiquitously detected followed by ALR. Concentration of pharmaceuticals ranged from 1 to 1000 ng/L, while the concentration for other classes ranged from 0.1 to 1000 ng/L. It is also observed that the concentration of TrOCs is usually greater than the predicted no-effect concentration (PNEC) of 10 ng/L.

Dilution and adsorption are the two natural attenuation process in oceans. However, concentration of a few TrOCs such as ketoprofen, gemfibrozil and caffeine can be higher than PNEC, suggesting that dilution could not reduce the concentration of TrOCs to acceptable levels. For instance, concentration of gemfibrozil (77–758 ng/L) and ketoprofen (185–805 ng/L) was higher than their PNEC value of 10 ng/L in the coastal waters of Costa Rica.

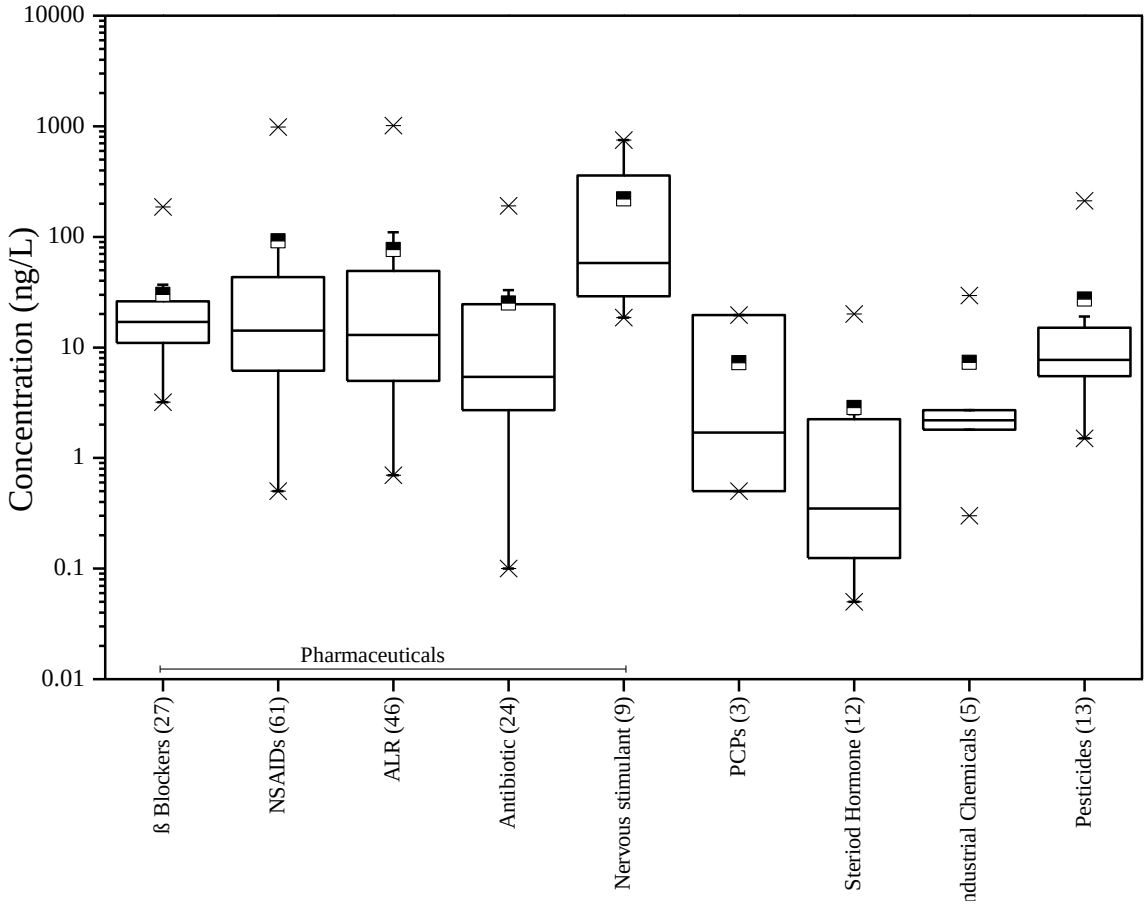

Figure 2.4. Average concentration (ng/L) of the five major classes of TrOCs in sea and coastal waters. Box plots represents interquartile range, median (horizontal line), min and max (whiskers), and average (black and white square box). Number of data points for each class/subclass is given in brackets on the x-axis. NSAIDs: Nonsteroidal anti-inflammatory drug; ALR: Anticonvulsant and Lipid regulator; and PCPs: Personal care products. Data is extracted from [103-108].

Adsorption of TrOCs in marine environment is influenced by acid dissociation constant (pKa) and pH of the seawater because these factors can change the octanol-water partition coefficient (log K_{ow}) of TrOCs. For instance, log K_{ow} of propranolol and fluoxetine was observed to increase linearly with pH [109, 110]. Similarly, typical pH=8 of seawater facilitates the lipophilicity of ionisable TrOCs onto marine sediments and marine organisms. For instance, McEneff et al. [111] reported incomplete dissociation of trimethoprim (pKa= 6.6) in marine environment. In some studies, log D has been used to predict the adsorption/sorption of TrOCs onto marine sediments/biota. For example, Fu et al. [112] observed that TrOCs with high log D (>3) are more likely to adsorb onto marine sediments. Occurrence of a few commonly detected TrOCs onto marine sediments and marine biota is presented in **Table 2.3**.

Table 2.3. Occurrence of commonly detected TrOCs in marine sediments and marine biota [113].

Compounds	Marine sediments (ng/g)	Marine biota (ng/g)
17β – estradiol	11–63	2.9–11.4
17α – ethinylestradiol	0.15–130	3– 38
Trimethoprim	0.1–734000	0.6
Tetracycline	0.6–7.1	1.9–9.5
Sulfamethoxazole	0.4–820000	2.3-20
Diclofenac	0.1–10	1.3–5.3
Naproxen	0.6–15.8	-
Ibuprofen	98–100	-
Acetaminophen	0.1–25.5	-
Atenolol	0.1–0.3	0.3–13
Propranolol	0.1–0.9	-
Clofibric Acid	0.1–0.1	
Gemfibrozil	0.1–0.9	-
Caffeine	1.9–12.2	-
Simvastatin	2–4	-
Triamterene	0.3–10.8	-
Carbamazepine	0.1–88.8	-

"-": not reported

2.3. TrOC removal by biological treatment processes

As discussed above in **Section 2.2**, TrOCs are ubiquitously detected in municipal wastewater, industrial wastewater, hospital wastewater and landfill leachate. Due to their ineffective removal by WWTPs (**Table 2.1**), widespread occurrence of TrOCs has been reported in freshwater bodies [46-54]. Concentrations of TrOCs in the effluent of WWTPs and sewage-impacted waterbodies range between hundreds of nanogram per litre (ng/L) and tens of microgram per litre (μg/L) [114, 115]. Even at the reported trace concentrations, several categories of TrOCs (*e.g.*, pharmaceuticals) are biologically active compounds, and cause acute and/or chronic ecological risks such as: (i) interference with the endocrine systems of the aquatic life; and (ii) accumulation in plants and animals. Thus, effective removal of TrOCs, particularly from municipal wastewater is essential for safe disposal of WWTP effluents.

Among different physicochemical and biological treatment processes investigated over the last few decades [116, 117], biological treatment processes, particularly conventional activated sludge (CAS) and membrane bioreactor (MBR) have been by far the most commonly investigated processes. Physicochemical treatment processes such as coagulation-flocculation, advanced oxidation processes (AOPs) and membrane separation processes (*e.g.*, nanofiltration, forward osmosis, and membrane distillation) have also been assessed for TrOC removal [16, 118].

Biodegradation is the dominant mechanism of TrOC removal in CAS-based biological process. During biological treatment, microorganisms convert and/or biomineralize TrOCs into simple organic and/or inorganic molecules (carbon dioxide and water). Microorganisms generate energy by utilizing organic compounds as a primary source of food. A part of this energy is used by the microorganisms for their cell growth, and the remaining energy is used for cell maintenance [119]. Since some TrOCs such as antibiotics and pesticides can be toxic to microorganisms, an additional growth substrate may be required to maintain microbial growth and diversity for adequate biodegradation. This process of adding an additional substrate is known as 'cometabolism' [120, 121]. In this section, performance of biological processes (such as conventional- and HR-MBRs) are discussed, and the factors governing the removal of TrOCs is critically analysed. This section is critical to understand the fate of TrOCs during biological treatment, and it facilitates in identifying the research gaps that led to the development of enzymatic-MBRs

2.3.1. TrOC removal by CAS process

In the CAS process, growth of microorganism takes place in an aeration tank under aerobic conditions. Among other conventional biological processes, the CAS process is the most widely applied treatment process for the treatment of municipal wastewater [122]. Although CAS-based WWTPs have not been designed for effective TrOC removal, it can achieve above 80% removal for some specific groups TrOCs such as bisphenol A, caffeine and surfactants. Several different mechanisms such as photolysis, volatilization, biodegradation and sorption onto activated sludge have been reported to contribute in TrOC removal, but major portion of these compounds are removed *via* biodegradation. The extent of TrOC removal depends on their physicochemical properties of TrOCs and operational parameters of the CAS process [123, 124]. This aspect is comprehensively discussed in **Section 2.3.3.4**. Performance of the CAS process for the removal of 86 TrOCs categorized based on their use/class is presented in **Figure 2.5**, indicating a wide range of variation.

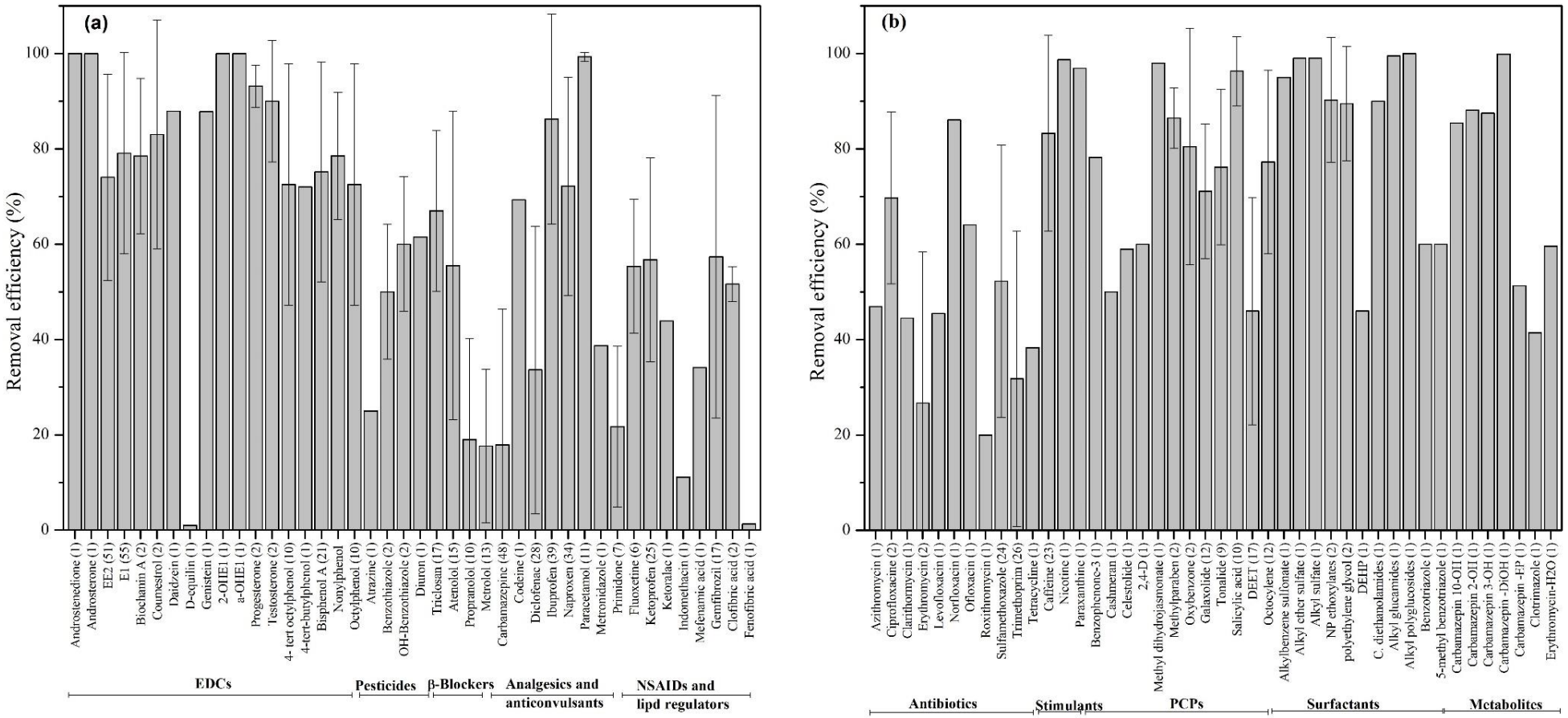

Figure 2.5. Removal of different categories of TrOCs by an CAS-based treatment process. Data presented as average ±standard deviation. Data is extracted from [125, 126].

The CAS process can achieve greater than 95% removal for all the selected surfactants. Effective removal of surfactant by the CAS process has been attributed to both sorption onto the activated sludge and biodegradation [122]. Margot et al. [127] reported that biodegradation remained the dominant mechanism of removal for surfactants. However, notably, they observed that sorption is mainly responsible for the removal of a cationic surfactant, namely dialkyldimethylammonium chloride [127].

All the EDCs are shown separately in **Figure 2.5**, because they can be harmful even a very low concentration. Of the 23 EDCs, complete elimination of four EDCs, namely 2-OHE1, a-OHE1, androstenedione and androsterone has been reported following treatment with the CAS process. Average removal of the remaining EDCs ranges from 75 to 90% except for the one syenthetic estrogen D-equilin (less than 5%) [128]. Due to their simple chemical structure, high sorption potential and biodegradability, EDCs are either adsorbed onto activated sludge or biodegraded by the activated sludge [123, 129]. According to the available literature, availability of dissolved oxygen is one of the key factors for effective EDC removal. For instance, Furuichi et al. [130] reported better removal of EDCs under aerobic conditions as compared to anaerobic conditions.

The CAS process has been reported to achieve significant removal (70-95%) for several PCPs such as methyl dihydrojasmonate, methylparaben, oxybenzone, galaxolide, tonalide and salicylic acid. Although removal of DEET, cashmeran, celestolide and 2,4-D is not as high as others TrOCs in this category, the CAS process can still achieve up to 60% removal. Notably, sorption was reported to be the dominant mechanism for PCPs with above 70% removal in the CAS process. Indeed, Margot et al. [127] estimated that the contribution of sorption in the overall removal of two PCPs (galaxolide and tonalide) can be as high as 80%. Removal of pesticides in the CAS process has been reported to range between 50 and 60% (**Figure 2.5**). Among the selected pesticides, atrazine is the most recalcitrant pesticide and was poorly removed (25%) by the CAS process (**Figure 2.5**).

Reported removals for different classes of pharmaceuticals such as beta-blockers, NSAIDs and antibiotics indicate that there is no apparent correlation in their removal. Beta-blockers such as atenolol and propranolol were poorly removed (<25 %) by the CAS process. Although atenolol and propranolol contain an electron donating group (EDG), their hydrophilic nature and poor affinity for biosolids could be the reason of their poor biodegradation in CAS process [124]. Among analgesics and anticonvulsants pharmaceuticals (**Figure 2.5**), ibuprofen, naproxen, and paracetamol were well removed (>70%) by the CAS process, probably because their chemical structure contains strong EDGs. Similarly, removal of a few antibiotics, namely ciprofloxacine, norfloxacin and ofloxacin as well as a few NSAIDs such as gemfibrozil and clofebric acid has been reported to be ranged between 50-60% in the CAS process. All nervous simulants and metabolites were observed to be completely removed (95-99 %) in the CAS process (**Figure 2.5**). In general, highly polar pharmaceuticals containing EWGs such as carbamazepine,

diclofenac, primidone and erythromycin are resistant to the biodegradation by the activated sludge in the CAS process and cannot be biodegraded by microorganisms. In addition, these TrOCs have the tendency to inhibit microbial activity [122, 131, 132]. Although it is difficult to classify the TrOC removal based on different categories, removal trends (**Figure 2.5**) can be written as follows: surfactants > nervous stimulants > metabolites > EDCs > personal care products > pesticides > pharmaceuticals except metabolites and stimulants.

2.3.2. TrOC removal by MBR

Membrane bioreactor (MBR) is considered as an effective improvement of the CAS process due to its robust and compact design as well as excellent effluent quality [133-135]. In conventional MBR, activated sludge is responsible for the degradation of the pollutants such as bulk organics, nutrients and TrOCs, while micro- or ultra-filtration (MF/UF) based membrane separation process effectively retains the activated sludge within the bioreactor [136-138]. Conventional MBR can achieve efficient aqueous phase removal of bulk organics from wastewater [139-141]. As far as TrOC removal is concerned, MBR may achieve better removal for certain groups of TrOCs as compared to the CAS process, possibly due to high concentration of the mixed liquor suspended solids (MLSS) and long solids retention time (SRT) [16, 124, 142]. MBR has been investigated extensively for the removal of TrOCs in the last decade, and its performance for the removal of TrOCs is shown in **Figure 2.6**. TrOCs are arranged in their respective class such as EDCs, pesticides and antibiotics. It is interesting to note that removal of some TrOCs such as fenoprop, triclosan, atenolol and carbamazepine varies from one study to another possibly due to different operating and environmental conditions.

Like the CAS process (**Figure 2.5**), average removal efficiency for EDCs is generally high and ranges from 80 to 99% in MBR. Above 90% removal has been observed to be achieved by MBR for two PCPs namely salicylic acid and octocrylene, while the removal for other personal care products range between 50 and 70%. As expected, pesticides have not been effectively removed by MBR except for triclosan and 2, 4-D. Atrazine was the most poorly removed compound with removal of less than 25% among all pesticides, while triclosan removal could be as high as 99% (**Figure 2.6**). Although different classes of pharmaceuticals showed TrOC-specific removal, removal of beta blockers and NSAIDs range from 70 and 90%. Some pharmaceuticals such as carbamazepine, trimethoprim and roxithromycin are poorly removed (<50%) in MBR. Compared to the CAS process (**Figure 2.5**), approximately 10-20% improvement in the removal of pharmaceuticals could be achieved in MBR.

Similar to other biological processes, removal of TrOCs in biological systems is also influenced by operational parameters and physicochemical properties of TrOCs such as chemical structure and hydrophobicity [124] as explained in **Section 2.3.3.4**. General trend in MBR based on different TrOC categories is as follows: EDCs > stimulants > NSAIDs > Analgesics > PCPs >

Antibiotics > beta-blokers > pesticides. Despite the effectiveness of MBR for the removal of a broad spectrum of TrOCs, knowledge is limited to understand the compound specific removal mechanisms and degradation pathways in addition to the fate of byproducts and their impacts on microbial communities [122, 124, 143, 144].

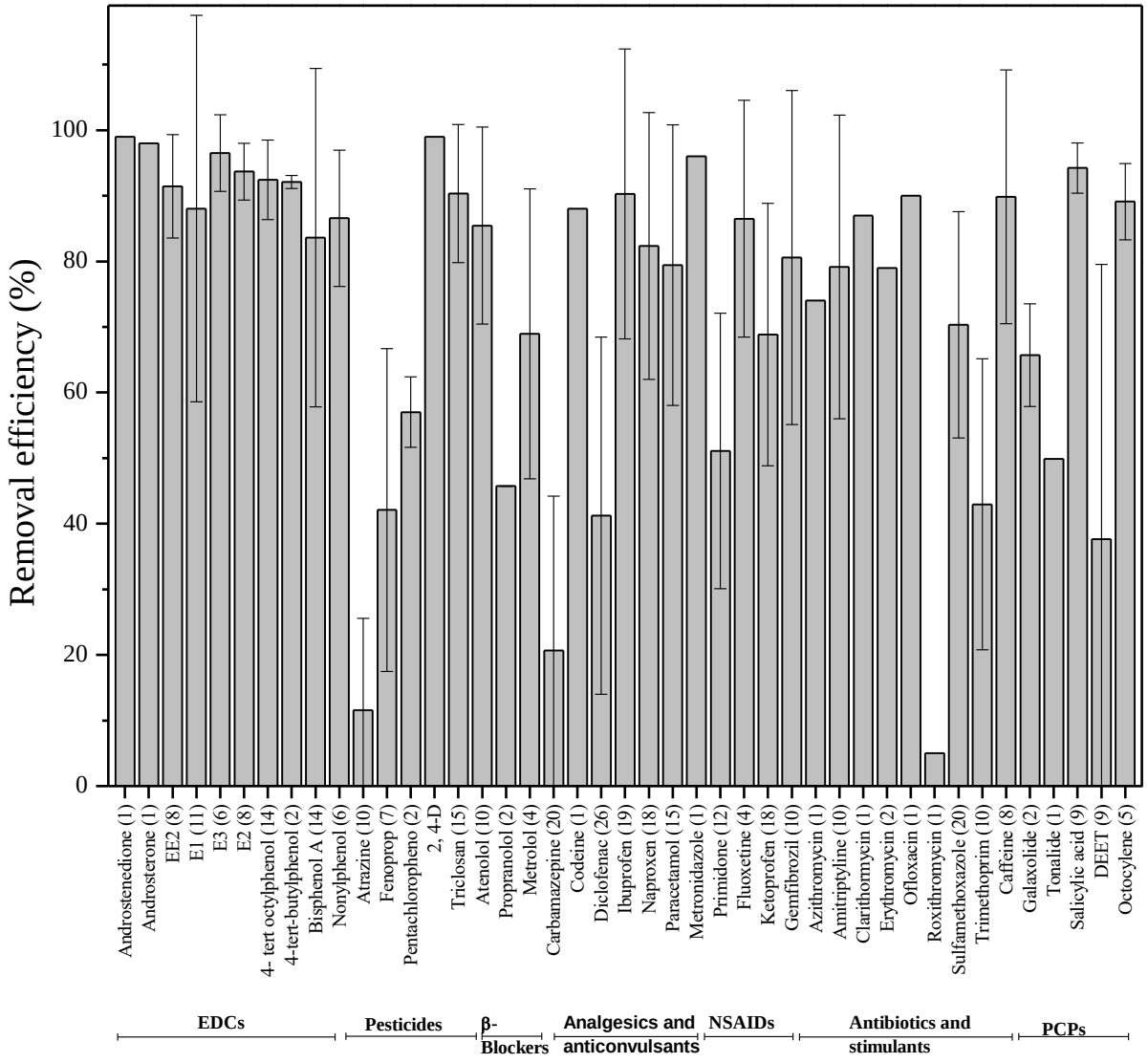

Figure 2.6. Removal of different categories of TrOCs by MBR. Data presented as average ±standard deviation. Data is extracted from [125, 126].

2.3.3. TrOC removal by high retention (HR)-MBR

Ineffectiveness of the CAS process and conventional MBR for the removal of certain groups of TrOCs is a significant concern. For effective removal of TrOCs, high retention membrane separation processes such as nanofiltration (NF)/reverse osmosis [145, 146] and membrane distillation [147-149] have been combined with conventional MBRs as a post-treatment step. To avoid an additional high retention membrane separation process, the high retention (HR)-MBRs have been developed, which can achieve TrOC retention by membrane and subsequent biodegradation in a single step for the production of high quality effluent suitable for water

reuse applications [150]. HR-MBR combines the high retention membranes such as nanofiltration (NF), forward osmosis (FO) or membrane distillation (MD) with an activated sludge process. Available studies report that HR-MBR provides effective removal of a wide range of TrOCs and can produce high quality TrOC-free effluent stream [151, 152]. One of the underlying rationales for the development of HR-MBR was that the effective retention of pollutants within the bioreactor may facilitate biodegradation due to the prolonged contact time between the activated sludge and TrOCs. Despite the effective TrOC retention by the high retention membranes [151, 153], degradation of TrOCs by activated sludge within the bioreactor has not been reported to consistently improve [151, 152]. This is because the degradation of TrOCs by the activated sludge depends on their intrinsic biodegradability that is governed by their physicochemical properties such as chemical structure and hydrophobicity [6].

A few excellent reviews on the main features, overall performance and technological constraints of HR-MBR have been published recently [150, 154-156]. However, removal of TrOCs by HR-MBR and factors affecting the removal of TrOCs by the activated sludge, particularly in HR-MBR have not been critically reviewed and discussed. This section aims to critically analyze the removal of TrOCs by the high retention membranes and activated sludge in HR-MBR. In addition, mechanisms of TrOC removal by HR-MBR are systematically elucidated. Based on the contribution of each mechanism of TrOC removal, a qualitative predictive framework is proposed.

2.3.3.1. HR-MBR configurations

In addition to the use of high retention membranes that allows effective retention of pollutants including TrOCs, HR-MBRs may have different features compared to the conventional MBR configuration (**Figure 2.7a**). Three configurations of HR-MBR, namely membrane distillation (MD)-MBR (**Figure 2.7b**), forward osmosis (FO)-MBR (**Figure 2.7c**) and nanofiltration (NF)-MBR (**Figure 2.7d**) have been investigated to-date [151, 152, 157].

Mechanisms of TrOC removal by HR-MBR include: (i) membrane retention; (ii) biodegradation; (iii) sorption; (iv) air stripping/volatilization; and (v) photolysis [6, 21, 158]. Removal of TrOCs by volatilization depends on the Henry's constant (H), which is the ratio of the concentration of a target pollutant in air to its concentration in wastewater. It has been reported that the removal of target pollutants *via* volatilization can be significant (5-10%) if their H values are higher than 0.005 [159-161]. Since the values of H for TrOCs generally fall in the range of 10^{-6} to 10^{-10}, TrOC removal in HR-MBR *via* volatilization is insignificant. Similarly, contribution of photolysis is negligible due to the high mixed liquor suspended solids (MLSS) concentration in the bioreactor [152, 162]. Hence, biodegradation, sorption and membrane retention mechanisms primarily contribute in varying extent for TrOC removal by HR-MBR as discussed in the following sub-sections.

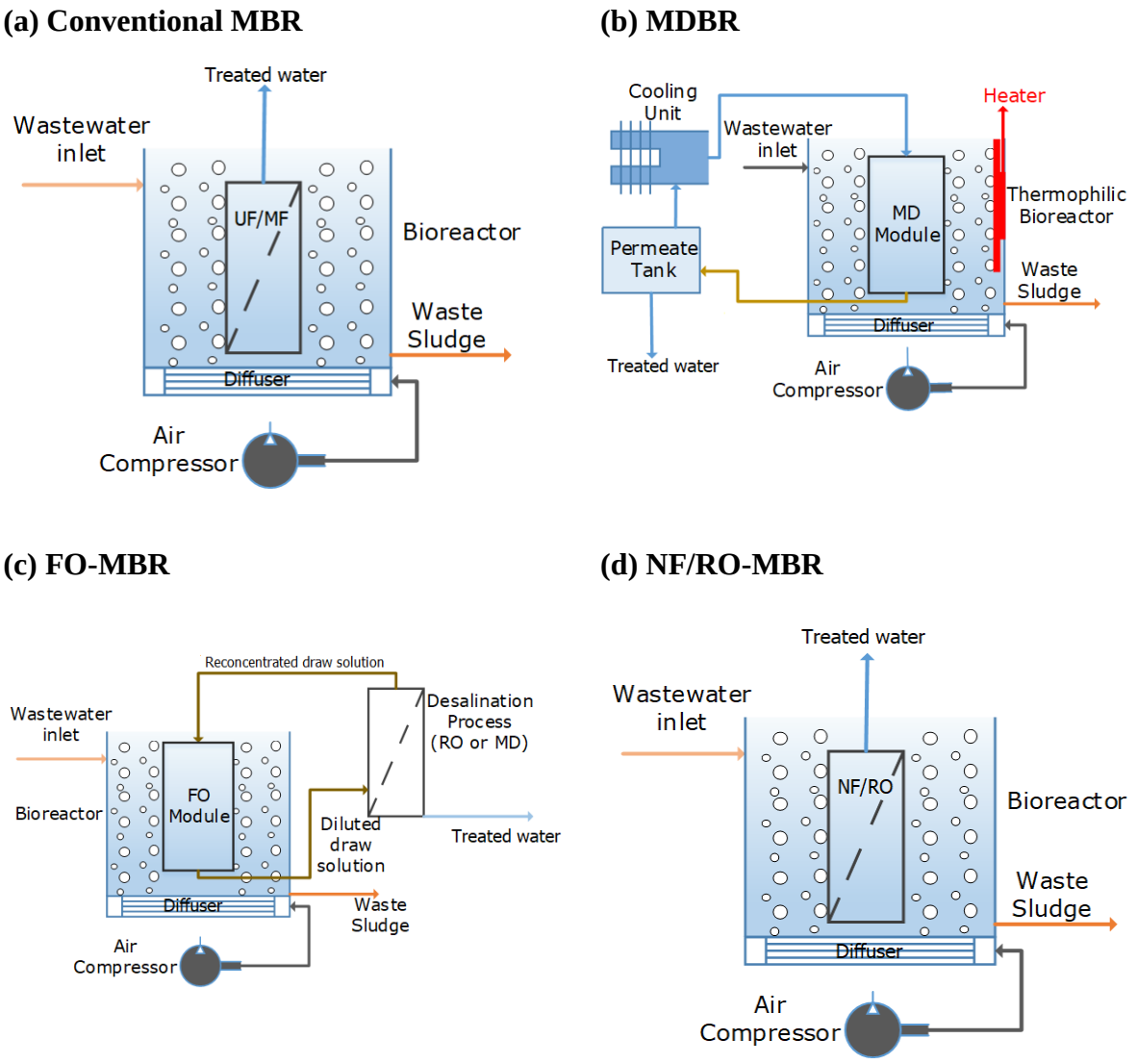

Figure 2.7. Schematics of (a) Conventional membrane bioreactor (MBR); (b) membrane distillation bioreactor (MDBR); (c); forward osmosis- membrane bioreactor (FO-MBR); and (d) nanofiltration- membrane bioreactor (NF-MBR)

2.3.3.2. Mechanisms of TrOC removal by high retention membranes

Retention by high retention membrane appears to be the most dominant mechanism for removal of TrOCs that are resistant to degradation by the activated sludge. Therefore, understanding the mechanisms of TrOC removal by MD, NF and FO membranes is vital. TrOC retention/removal by high retention membranes depends on: (i) the type of high retention membrane; (ii) influent characteristics; and (iii) operating conditions (**Table 2.4**). TrOC retention by NF and FO membranes has been reported to be influenced by a number of factors (**Table 2.4**) such as physicochemical properties (*e.g.*, hydrophobicity, charge and molecular weight) of TrOCs, operating parameters and membrane properties as explained below [163-166]. On the other hand, TrOC retention by MD membranes depends on the volatility (pK_H) and hydrophobicity (log D) of pollutants [147, 150], thereby making TrOC retention by MD

membrane simpler as compared to NF and FO membranes. In a stand-alone MD process, 'pK_H/log D' ratio of less than 2.5 corresponds to ineffective TrOCs retention (50-70%), while TrOCs with a high pK_H/log D ratio (>2.5) are effectively retained (90-99%) by MD membranes [147]

Table 2.4. Factors affecting the retention of TrOCs by high retention membranes

Factors	MD membrane	FO membrane	NF membrane
Fouling	*	*	*
Diffusion of solute	-	*	*
Hydrophobicity	*	*	*
Membrane MWCO	-	*	*
Charge on TrOCs	-	*	*
Membrane surface charge	-	*	*
Polarity	-	*	*
Molecular width	-	*	*
Volatility of TrOCs	*	*	*
Temperature and pH	*	*	*

" –" : no effect according to available reports

Mechanisms of TrOC retention by NF and FO membrane consist of: (i) the net sorption of a solute on the membrane surface; (ii) the transport of solute inside the membrane; and (iii) the sieving property of the membrane [150, 167, 168]. Influence of other factors including hydrophobicity and charge repulsion on sorption and solute transport has also been observed [169, 170]. In general, size exclusion mechanism is responsible for the retention of non-ionic and hydrophilic (log D <3) TrOCs, and the extent of retention depends on the molecular weight cut-off (MWCO) of membranes. For example, a tight NF membrane (MWCO <200 g/mole) achieved 97% retention of carbamazepine (log D = 1.89) from a filtered lake water containing a mixture of 22 TrOCs, while only 50% removal was observed by a loose NF membrane (MWCO >300 g/mole) [171]. In another study by Xie et al. [172], retention of carbamazepine by a cellulose triacetate FO membrane remained in between 80 and 90% at different pH values (*i.e.,* 3.5-7.5). Similarly, carbamazepine retention by cellulose triacetate and thin film composite polyamide FO membranes was reported to be 90-95% [173]. Effective retention (80-99%) of other hydrophilic TrOCs such as metronidazole (log D = -0.14), clofibric acid (log D = -1.06) and N, N-Diethyl-meta-toluamide (DEET, log D = 2.42) by NF and FO membranes has been reported [145, 174-177]. Hydrophobic TrOCs (log D >3) such as steroid hormones, bisphenol A and 4-tert-octylphenol have also been reported to be effectively retained (>80%) by both NF and FO membranes [164, 177, 178]. Notably, hydrophobicity of TrOCs can

influence their retention because hydrophobic TrOCs can adsorb onto the membrane surface, thus initially resulting in their effective retention. However, as the filtration continues, their retention may reduce due to their subsequent diffusion into the permeate [164, 177]. Compared to hydrophilic TrOCs, hydrophobic TrOCs, regardless of their size, can diffuse into the permeate to attain an equilibrium between the concentration of hydrophobic TrOCs on/near the membrane surface and the permeate. This gradually reduces the extent of TrOC retention by the NF and the FO membranes [179-181]. Once an equilibrium between the concentration of TrOCs on/near membrane surface and permeate is established, the role of adsorption in TrOC retention diminishes, and charge repulsion and size exclusion mechanisms govern the retention of TrOCs by NF and FO membranes [168, 182].

NF and FO membranes are negatively charged at pH=7 owing to the protonation of their functional groups [168, 171]. Hence, membrane surface charge and its interaction with charged TrOCs such as diclofenac, naproxen and ibuprofen will govern the extent of their retention. Poor rejection of positively charged hydrophobic TrOCs such as steroid hormones by NF/FO membrane can be attributed to the attraction between positively charged TrOCs and negatively charged membrane surface. This consequently increases the concentration of solute at the surface of membrane, thus increasing their diffusion into permeate. On the other hand, effective retention of negatively charged hydrophilic TrOCs is due to the charge repulsion mechanism, which keeps TrOCs away from the membrane surface [179, 183, 184]. Notably, the transformation of neutral TrOCs to negatively charged TrOCs at $pH>pK_a$ can improve their retention by NF and the FO membranes. For example, an increase of 50 and 65% in the retention of sulfamethoxazole (pK_a = 5.6) and ibuprofen (pK_a = 4.47), respectively, by a thin film composite NF membrane was observed when the feed pH was changed from 5 to 10 [167]. In another study, retention of ibuprofen (pK_a = 4.47) and naproxen (pK_a = 4.2) by an FO membrane was observed to be increased by 10-15% due to the increase in the pH of feed from 6 to 8 (*i.e.*, $pH>pK_a$) [173]. Based on the discussion regarding the factors affecting the retention of TrOCs by NF and FO membrane, a qualitative predictive framework is presented in **Figure 2.8**.

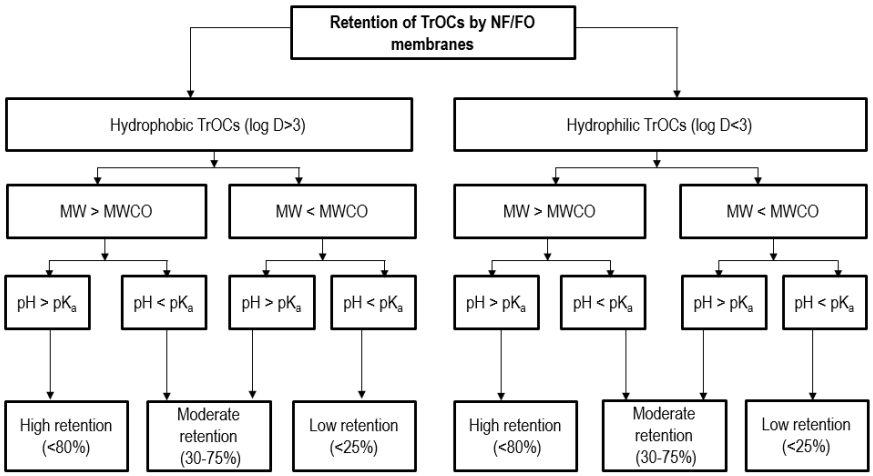

Figure 2.8. Qualitative predictive framework for the retention of TrOCs by NF or FO membrane. The case of an acidic compound is depicted here. A basic compound will become positively charged at pH<pk_b. The transformation of neutral TrOCs to negatively charged at pH > pK_a or positively charged at pH < pK_b can improve their retention by NF and the FO membranes. Modified from [166, 170]

2.3.3.3. Aqueous phase removal of TrOCs by HR-MBR

As mentioned before, three configurations of HR-MBR, namely membrane distillation bioreactor (MDBR), forward osmosis (FO-MBR) and nanofiltration (NF-MBR) have been investigated to-date [151, 152, 157, 185]. Depending on the physicochemical properties of TrOCs and the type of HR-MBR configuration, removal of TrOCs by HR-MBRs can range between 90-99% (**Table 2.5**).

The advantage of an integrated biodegradation and membrane separation process is that HR-MBR can achieve better TrOC removal as compared to the standalone HR-membrane. For instance, Wijekoon et al. [147] studied the rejection of a mixture of 30 TrOCs by a standalone MD process, and observed partial retention (50-70%) of a few volatile TrOCs (pK_H <9) such as 4-tert-octylphenol (pK_H= 5.06), benzophenone (pK_H= 5.88) and amitriptyline (pK_H= 8.18). On other hand, when the performance of MDBR was studied for the removal of a mixture of 30 TrOCs, effective removal (95-99%) was achieved by MDBR for all the selected 30 TrOCs including those partially removed by the standalone MD process [147, 152].

Compared to ineffective or unstable removal of a few hydrophobic TrOCs such as bisphenol A (40-80%), oxybenzone (70-75%), estrone (80%), and 17α – ethynylestradiol (70-90%) by a standalone FO process [168], FO-MBR has been reported to achieve above 99% removal for hydrophobic TrOCs [151, 186]. Better performance of MDBR and FO-MBR for TrOC removal as compared to the standalone MD and FO process can be attributed to the efficient degradation of volatile and hydrophobic TrOCs such as 4-tert-octylphenol, benzophenone, triclosan, bisphenol A and oxybenzone by the activated sludge [152, 186, 187].

Table 2.5. Physicochemical properties of TrOCs and their aqueous phase removal by HR-MBR

TrOCs	Chemical formula [a]	Molecular Weight [a]	Dissociation coefficient (pK_a) [a]	Henry constant (H) [b]	pK_H [b]	Log D at pH=7 [a]	Removal efficiency (%)		
		g/mole					FO-MBR [c]	MDBR [d]	NF-MBR [e]
Primidone	$C_{12}H_{14}N_2O$	218.25	12.26 ± 0.40	1.164E-14	13.93	0.83	>99	>99	-
Ketoprofen	$C_{16}H_{14}O_3$	254.28	4.23 ± 0.10	2.005E-14	13.70	0.19	>99	>99	94
Naproxen	$C_{14}H_{14}O_3$	230.26	4.84 ± 0.30	2.096E-13	12.68	0.73	>99	>99	98
Gemfibrozil	$C_{15}H_{22}O_3$	250.33	4.75	7.677E-13	12.11	2.07	>95	>99	99
Metronidazole	$C_6H_9N_3O_3$	171.15	14.44 ± 0.10	2.073E-12	11.68	-0.14	>95	>99	99
Diclofenac	$C_{14}H_{11}Cl_2NO_2$	296.15	4.18 ± 0.10	3.098E-12	11.51	1.77	>95	90	45-95
Fenoprop	$C_9H_7Cl_3O_3$	269.51	2.93	3.284E-12	11.48	-0.13	83-99	95	-
Ibuprofen	$C_{13}H_{18}O_2$	206.28	4.41 ± 0.10	4.066E-11	10.39	0.94	>99	>99	100
Ametryn	$C_9H_{17}N_5S$	27.33	3.71±0.41	4.418E-10	9.35	2.97	>99	>99	-
Clofibric acid	$C_{10}H_{11}ClO_3$	214.65	3.18 ±0.10	2.909E-10	9.54	-1.06	>99	>99	75
Carbamazepine	$C_{15}H_{12}N_2O$	236.27	13.94 ± 0.20	8.168E-10	9.09	1.89	50-99	95	18-75
Octocrylene	$C_{24}H_{27}N$	361.48	-	3.382E-09	8.47	6.89	80-90	90	95
Amitriptyline	$C_{20}H_{23}N$	277.40	9.18 ± 0.28	6.596E-09	8.18	2.28	>99	>99	83-100
Atrazine	$C_8H_{14}ClN_5$	215.68	2.27 ± 0.10	5.223E-08	7.28	2.64	75-90	>99	16-80
Propoxur	$C_{11}H_{15}NO_3$	209.24	1.49 ± 0.70	5.265E-07	6.28	1.54	>99	>99	-
Benzophenone	$C_{13}H_{10}O$	182.22	-	1.316E-06	5.88	3.21	>99	95	>99
N, N-Diethyl-meta-toluamide (DEET)	$C_{12}H_{17}NO$	191.3	-	1.410E-06	5.85	2.42	40-90	-	60
Estriol	$C_{18}H_{24}O_3$	298.33	10.25 ± 0.70	1.644E-11	10.78	1.89	>99	>99	-
17α – Ethynylestradiol	$C_{20}H_{24}O_2$	269.40	10.24 ± 0.60	3.399E-10	9.47	4.11	>99	>99	-
Oxybenzone	$C_{14}H_{12}O_3$	228.24	7.56±0.35	5.851E-10	9.23	3.89	>99	>99	-
Estrone	$C_{18}H_{22}O_2$	270.37	10.25 ± 0.40	9.286E-10	9.03	3.62	>99	>99	95
17β – Estradiol	$C_{18}H_{24}O_2$	272.38	10.27	1.173E-09	8.93	4.15	>99	>99	-
17β – Estradiol-17-acetate	$C_{20}H_{26}O_3$	314.42	10.26 ± 0.60	2.151E-09	8.67	5.11	>99	>99	-
Bisphenol A	$C_{15}H_{16}O_2$	228.29	10.29 ± 0.10	2.197E-09	8.66	3.64	>99	>99	95-97
Salicylic acid	$C_7H_6O_3$	138.12	3.01 ± 0.10	6.653E-09	8.18	-1.13	>99	95	70
Triclosan	$C_{12}H_7Cl_3O_2$	289.54	7.80 ± 0.35	6.537E-07	6.18	5.28	>99	>99	82
4-tert-Butylphenol	$C_{10}H_{14}O$	150.22	10.13 ± 0.13	7.136E-06	5.15	3.40	>99	>99	88
4-tert-Octylphenol	$C_{14}H_{22}O$	206.32	10.15 ± 0.15	8.670E-06	5.06	5.18	>99	>99	-

[a] Data extracted from SciFinder Scholar; [b] Henry's law constant (H) = Vapour pressure × molecular weight/water solubility; and $pK_H = - \log_{10} H$. [c] Wijekoon, Hai, Kang, Price, Guo, Ngo, Cath and Nghiem [152]; [d] Alturki, McDonald, Khan, Hai, Price and Nghiem [188]; Holloway, Regnery, Nghiem and Cath [187]; Lay, Zhang, Zhang, McDougald, Tang, Wang, Liu and Fane [189] Luo, Hai, Kang, Price, Nghiem and Elimelech [186]; and Luo, Phan, Xie, Hai, Price, Elimelech and Nghiem [151]; [e] Phan, McDonald, Hai, Price, Khan, Fujioka and Nghiem [157]; and Wang [153]
"–": not available

Both MDBR and FO-MBR was reported to achieve effective removal of a range of TrOCs (Table 2) [150, 190]. Indeed, a comparison of the aqueous phase removal of TrOCs by CAS, conventional MBR and HR-MBR reveals that median TrOC removal by HR-MBR is almost 90%, while median values for CAS and MBR are approximately 60 and 65%, respectively (**Figure 2.9**).

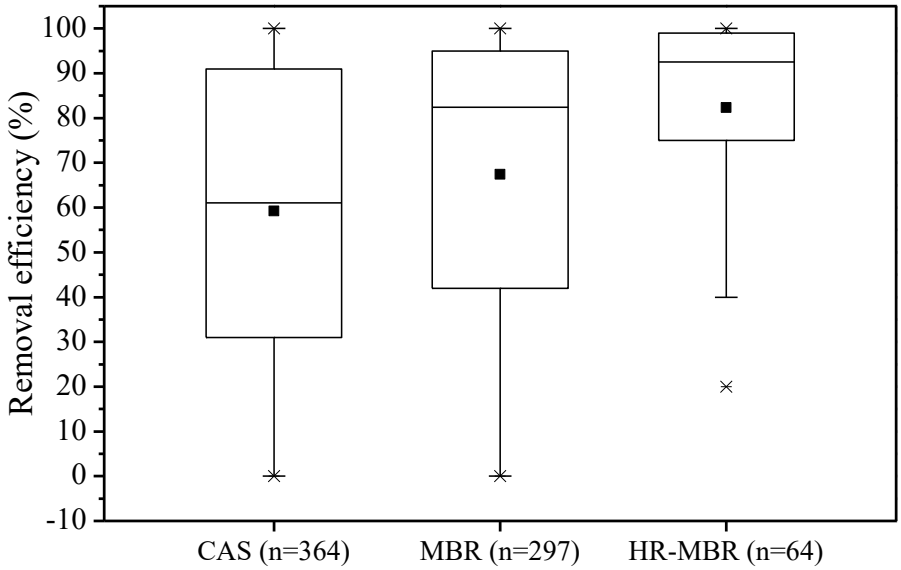

Figure 2.9. Aqueous phase removal of TrOCs by CAS, MBR and HR-MBR. Box-and-whisker plot is showing information about: the interquartile range; median (horizontal line in the box); min and max (whiskers); and average (block square in the box). Adapted from [126].

2.3.3.4. Factors affecting TrOC removal by activated sludge in HR-MBR

2.2.3.4.1. Effect of TrOC molecular structure

Degradation of TrOCs by activated sludge depends on their intrinsic biodegradability and sorption potential. The extent of TrOC degradation can vary depending on the chemical structure of the target compound [186, 191]. In general, simple structured TrOCs without branched/multi chain alkyl groups are readily biodegraded compared to structurally complex TrOCs due to their resistance to microbial degradation. Similar to conventional MBR, TrOCs containing strong electron withdrawing functional groups (EWG) such as carboxyl, halogen and amide are resistant to biodegradation, and their degradation is also poor and/or unstable in HR-MBR [152, 157]. For instance, atrazine, carbamazepine and diclofenac are resistant to biodegradation due to the presence of EWGs (*i.e.*, halogen and amide) in their structures [191, 192].

Based on their biodegradation, TrOCs can be divided into three categories: (i) low or unstable removal (5-30%) for TrOCs containing strong EWGs such as atrazine, carbamazepine and primidone; (ii) consistently high removal (80-90%) of hydrophobic TrOCs containing electron donating groups (EDGs) such as steroid hormones; and (iii) poor to moderate removal (30-80%)

of hydrophilic TrOC containing both EWGs and EDGs [152, 157, 193]. Limited degradation of some TrOCs by the activated sludge highlights the significance of high retention membranes in effective TrOC removal for producing a high-quality effluent. Specific groups of TrOCs that are poorly degraded by the activated sludge accumulate within the bioreactor of HR-MBR. Thus, there is a need to introduce microbes such as fungi with strong degradation capacity.

2.2.3.4.2. Effect of TrOC sorption on activated sludge

Hydrophobic TrOCs (log D>3) can adsorb onto the activated sludge by following mechanisms: (i) chemical binding to bacterial proteins and nucleic acids; (ii) sorption onto polysaccharide structures outside the bacterial cell; (iii) adsorption onto bacterial lipid structure [129]. With a few exceptions, HR-MBR can achieve as high as 99% removal of hydrophobic TrOCs *via* biodegradation and sorption [152, 187]. Additionally, non-hydrophobic interactions such as hydrogen bonding, electrostatic interactions and ion exchange can also instigate sorption of hydrophilic TrOCs onto activated sludge. For instance, Wijekoon et al. [152] observed that sorption significantly contributed to the removal of a hydrophilic TrOC salicylic acid (log D = -1.22).

Sorption on activated sludge contributes to improvement of overall aqueous phase removal of TrOCs in conventional MBRs [160, 189, 194-196]. For instance, halogenated TrOCs are widely reported to be persistent to microbial degradation. However, the increase in halogen-content increases the hydrophobicity of halogenated TrOCs [197]. Thus, efficient removal of halogenated TrOCs, particularly of triclosan, have been reported to be achieved by even conventional MBRs due to its sorption onto activated sludge [6, 191, 198]. Although sorption also contributes to the removal of TrOCs within the bioreactor of HR-MBRs, the overall TrOC removal by HR-MBR is less dependent on sorption because of the high retention membranes, which can retain even the TrOCs demonstrating low sorption on sludge.

Following sorption onto the activated sludge, the extent of TrOCs degradation depends on their intrinsic biodegradability [6]. For instance, Wijekoon et al. [152] observed higher concentrations of two highly hydrophobic TrOCs, namely triclosan and octocrylene in the sludge samples of an MDBR as compared to other hydrophobic TrOCs such as bisphenol A and steroid hormones. This is because of the presence of strong EWGs in the molecular structure of triclosan and octocrylene *i.e.*, halogen and carbonyl, respectively [6, 191].

2.2.3.4.3. Effect of mixed liquor suspended solids concentration

Conceptually, mixed liquor suspended solid (MLSS) concentration may affect the removal of TrOCs in a biological process by influencing the rate of biodegradation. However, biodegradation also depends on TrOC physicochemical properties and diversity of microbial communities [199-

201]. Indeed biodegradation of TrOCs containing EDGs in their molecular structure (*i.e.,* easily biodegradable) has been reported to be 80-99% in conventional MBRs at the tested MLSS concentrations ranging from 2-15 g/L [202-205]. Similarly, effective degradation (90-99%) of TrOCs containing EDGs such as naproxen, ketoprofen, bisphenol A and t-octylphenol has been achieved in NF-MBR, FO-MBR and MDBR over MLSS concentrations of 2-5 g/L [151, 152, 157]. Holloway et al. [187] also achieved 95-99% degradation of TrOCs containing strong EDGs such as naproxen, oxybenzone, ibuprofen and caffeine by operating an FO-MBR at a MLSS concentration of 3-4 g/L.

Degradation of hydrophilic TrOCs containing EWGs in conventional MBR has been reported to be poor irrespective of operating MLSS concentrations [199, 206-209]. Similarly, poor and unstable degradation (15-40%) by the activated sludge in HR-MBR has been reported for hydrophilic TrOCs containing EWGs such as carbamazepine, DEET and atrazine [157, 186].

2.2.3.4.4. Effect of solids retention time

Solids retention time (SRT) governs the microbial makeup of a bioreactor. Conceptually, long SRT may improve the extent of TrOC removal by providing adequate time for the development of special TrOC degrading microbial communities [200, 210, 211]. Indeed, biodegradation of a few resistant TrOCs such as sulfamethoxazole, diclofenac, mefenamic acid and carbamazepine improved significantly following an increase in the SRT of conventional MBR (**Figure 2.10**). The biodegradation of resistant TrOCs containing strong EWGs varied depending on the type of HR-MBR configuration. For instance, FO-MBR (SRT = 20 days) achieved better degradation of carbamazepine, atrazine, clofibric acid, fenoprop and diclofenac as compared to MDBR (SRT = 88 days) [151, 152, 157]. Disrupted metabolic activities associated with the treatment in thermophilic conditions may have resulted in less effective degradation of resistant TrOCs by MDBR [120, 152]. However, a systematic study is necessary to determine the actual reasons of these observations.

As expected, no improvement was observed in the degradation of easily biodegradable TrOCs containing EDGs such as naproxen, ketoprofen and ibuprofen by increasing the SRT of a conventional MBR beyond 15 days [198, 204, 212, 213]. Similarly, no observable effect of SRT on the degradation of TrOCs such as naproxen, ketoprofen, ibuprofen, bisphenol A and 4-tert-octylphenol has been reported in HR-MBRs over a wide range of SRTs [152, 157, 187, 214].

2.2.3.4.5. Effect of operating temperature

To date lab-scale FO- and NF-MBRs have been operated at the room temperature *i.e.*, 18-21 ºC, while the operating temperature of MDBR falls in the thermophilic range *i.e.*, 40-60 ºC [152, 157, 215, 216]. As noted in the previous section, relatively less degradation of a few hydrophilic TrOCs

such as carbamazepine, atrazine, clofibric acid, fenoprop and diclofenac has been observed in MDBR as compared to FO-MBR [151, 152]. This can be attributed to the higher operating temperature of MDBR, which can disrupt microbial activities. Particularly, high operating temperature (>35 ºC) can affect TrOC degradation by reducing the abundance of nitrifying bacteria [217-219]. In conventional MBR, improvement in TrOC removal has been reported to concur with the achievement of efficient nitrification [220]. To provide further insight into this aspect, the effect of thermophilic conditions on the microbial diversity and TrOC removal in various formats of HR-MBR should be further investigated.

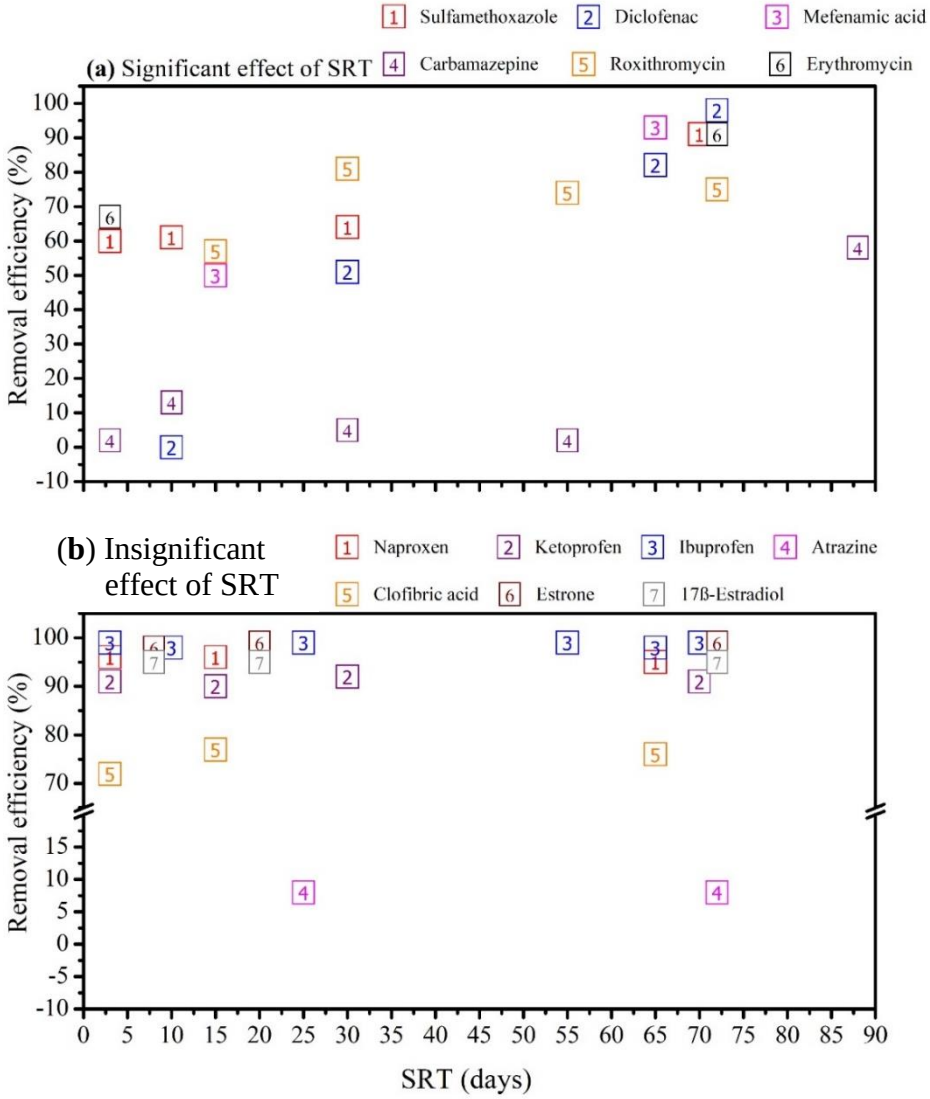

Figure 2.10. Effect of SRT on the aqueous phase removal of selected TrOCs by conventional MBR. (**a**) Significant SRT dependent improvement in TrOC removal; and (**b**) insignificant dependence of TrOC removal on SRT. Adapted from [126].

2.3.3.5. Fate of TrOCs in HR-MBR

Effective retention of TrOCs (90-99%) within the bioreactor of HR-MBR by the high retention membranes may facilitate their biodegradation due to the prolonged contact time between the activated sludge and TrOCs. Indeed, comparing data from independent studies, degradation of some TrOCs seems to be more stable in HR-MBR as compared to conventional MBR and CAS (**Figure 2.11**).

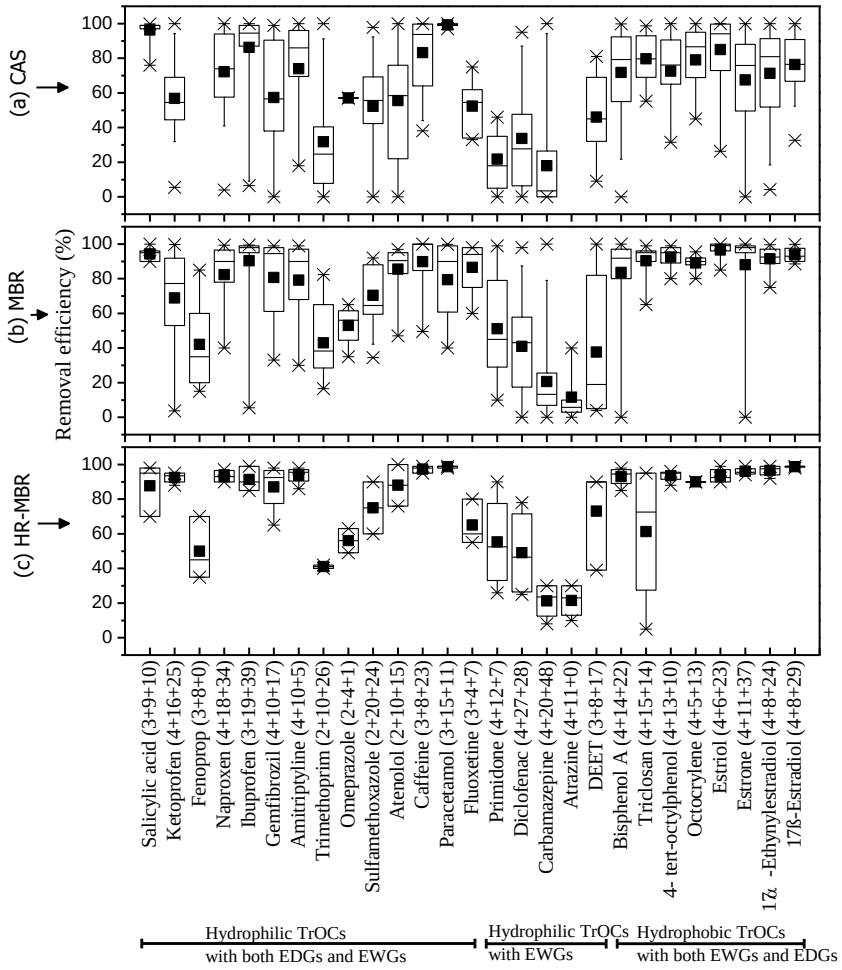

Figure 2.11. Variations in the biodegradation of TrOCs in CAS (a), MBR (b) and HR-MBR (c). Box-and-whisker plot is showing information about: the interquartile range; median (horizontal line in the box); min and max (whiskers); and average (block square in the box). Numbers in the parenthesis on the x-axis represent the no. of data points (no. of data points: HR-MBR+MBR+CAS). Adapted from [126].

The degradation improvement for these TrOCs in HR-MBR is discernible, however, not very high. An assessment of the relative contribution of different mechanisms of TrOC removal suggests that membrane retention and biodegradation govern the effectiveness of treatment by HR-MBR (**Figure 2.12**). According to the available literature, TrOC removal in HR-MBR *via* sorption onto

activated sludge ranges between 1-10% and 2-30% for hydrophilic and hydrophobic TrOCs, respectively.

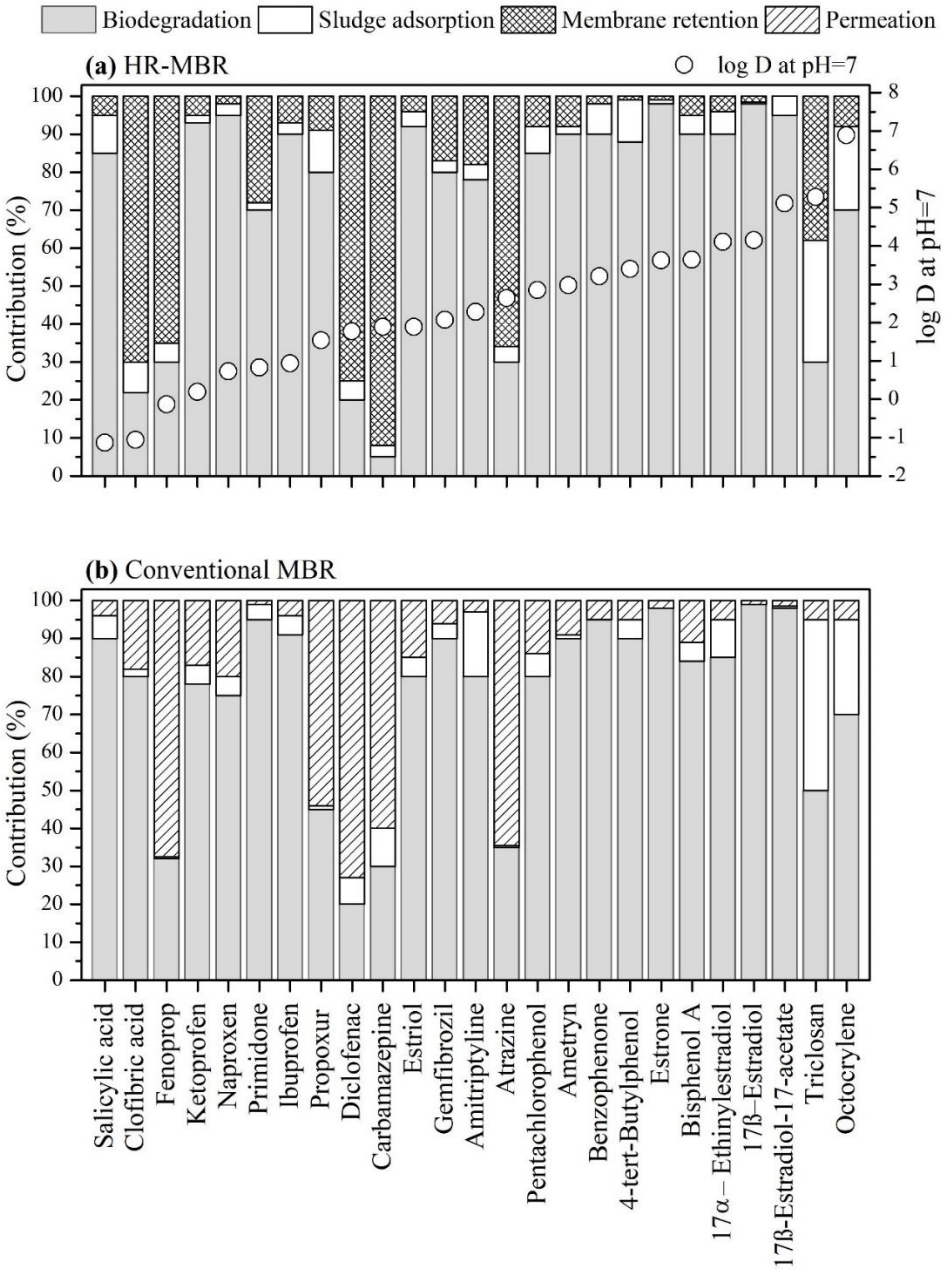

Figure 2.12. Contribution of different mechanisms for TrOC removal in HR-MBR and conventional MBR. HR-MBR. Adapted from [126].

The fate of TrOCs during wastewater treatment by HR-MBR is governed by the TrOC physicochemical properties (*e.g.*, chemical structure and hydrophobicity), which influence their biodegradation. The hardly biodegradable TrOCs will not appear in the treated effluent because of the extra barrier provided by the high retention membranes. However, when not subsequently

biodegraded, their accumulation on sludge would complicate sludge disposal and reuse. Based on the contribution of each mechanism of TrOC removal, a qualitative framework for the removal of TrOCs in HR-MBR is proposed in **Figure 2.13**.

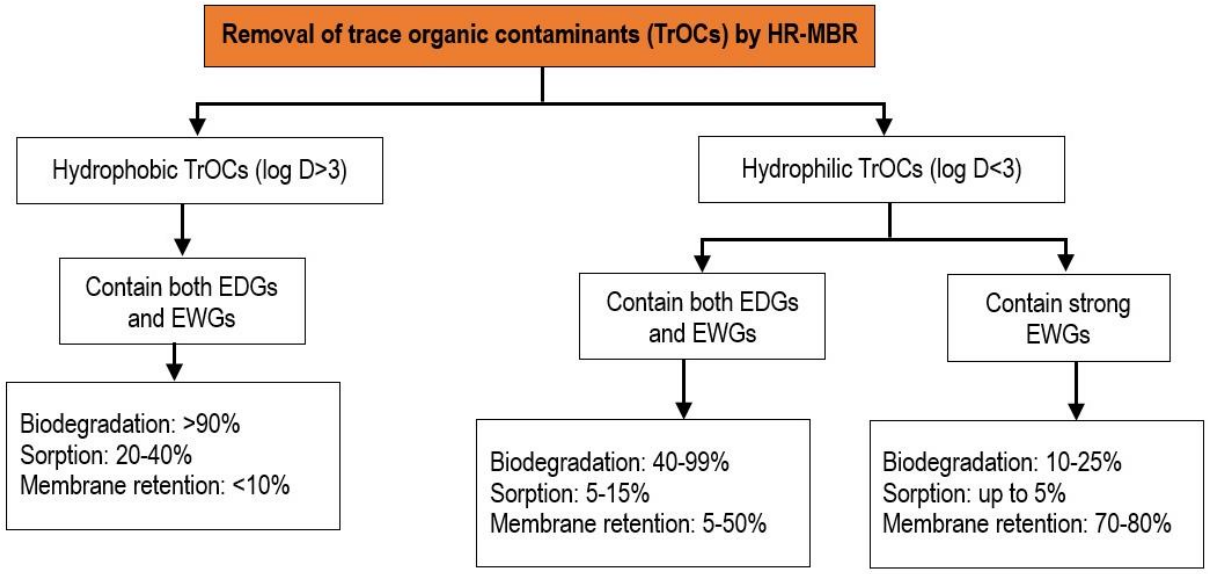

Figure 2.13. A qualitative framework to predict the contribution of different mechanisms of TrOC removal in HR-MBR categorized based on their physicochemical properties.

An in-depth assessment of the available literature on HR-MBR performance suggests that, as compared to CAS and conventional MBRs (using micro- or ultrafiltration membrane), aqueous phase removal of TrOCs in HR-MBR is significantly better. However, a strong evidence of improvement in TrOC degradation in HR-MBR is not available. In fact, poor degradation of resistant TrOCs HR-MBR leads to their accumulation within the bioreactor of HR-MBR. To improve the degradation of TrOCs in HR-MBR, other microbes with better TrOC degradation capacity than the conventional activated sludge can be introduced. In this context, white-rot fungi and their extracellular enzymes [221] are worth-noting. White-rot fungi (WRF) and their enzymes have been reported to achieve effective degradation of TrOCs that are resistant to activated sludge-based treatment process [222-224]. Performance of white-rot fungi and their enzymes for the degradation of a wide range of TrOCs is elucidated in the next section.

2.4. TrOC degradation by white-rot fungi (WRF)

White-rot fungi (WRF) are a type of fungus that can degrade lignin, a class of complex natural organic polymers found in the cell wall of plants, by using their extracellular enzymatic system, called as ligninolytic enzymes [225, 226]. WRF and their ligninolytic enzymes have also been studied for the treatment of a variety of recalcitrant compounds such as polycyclic aromatic hydrocarbons, dyes, and chlorophenols [222, 227, 228]. In particular, removal of TrOCs using WRF or their extracellular enzymes has gained much attention over the last decade [229-231]. TrOCs such as pharmaceuticals, personal care products, industrial chemicals and steroid hormones have been commonly detected in municipal wastewater and surface water bodies as discussed in **Section 2.2**. Their occurrence in environmental systems can be harmful to aquatic ecosystem and human health even at trace concentrations [5, 16].

Whole-cell WRF and their ligninolytic enzymes have been reported to efficiently remove a wide range of TrOCs such as pharmaceuticals (*e.g.*, ibuprofen, ketoprofen and diclofenac), personal care products (*e.g.*, triclosan and oxybenzone) and steroid hormones [229, 232-234]. Moreover, a number of influencing factors for such treatment systems have been identified. These factors include physicochemical properties of TrOCs, type of WRF species and their individual ligninolytic extracellular enzymes as well as culture medium and environmental conditions [222, 235]. The potential of WRF for the removal of TrOCs has been investigated mostly in batch mode. There are only a few studies on continuous-flow reactor configurations [234, 236-239]. Despite a significant research effort, efficient removal of TrOCs by WRF mediated treatment, their removal mechanisms and degradation pathways remain largely to be elucidated. There have been a few excellent reviews on bioremediation of recalcitrant compounds by WRF and their enzymes [222, 235, 240-242]. In this section, performance of WRF and their ligninolytic enzymes as well as the performance governing factors is critically analysed and discussed. In addition, the performance of conventional enzymatic membrane bioreactor (EMBR) is elucidated. The key research gaps are identified, indicating the need of high retention (HR)-EMBR.

2.4.1. Properties of WRF and their ligninolytic enzymes

WRF species degrade recalcitrant compounds including TrOCs by using their intracellular or extracellular enzymes [222, 243]. The key features of WRF that make them an attractive treatment option for TrOC removal include but are not limited to (i) the non-specificity and non-selectivity of their enzyme systems, enabling them to degrade complex individual and mixture of pollutants; (ii) the secretion of extracellular enzymes, enabling them to degrade pollutants with low water solubility; (iii) the ability of their plasma membrane-dependent redox system to degrade pollutants in a nutrient deficient reaction mixture over a wide range of pH; and (iv) the ability of intracellular enzyme to degrade some pollutants [225, 244, 245]. Depending on growth medium and culture

conditions as well as on the type of WRF species/strains, WRF can secrete four different ligninolytic enzymes namely laccase, lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP). In addition, cytochrome P450 monooxygenases, a group of intracellular enzymes, have also been reported to play a vital role in the degradation of TrOCs *via* hydroxylation, dehalogenation and heteroatom oxygenation mechanisms [222, 246, 247].

Characteristics of ligninolytic extracellular enzymes such as molecular mass, isoelectric point and redox potential are outlined in **Table 2.6**. Stability and catalytic potential of ligninolytic enzymes may vary due to difference in their redox-potential as well as due to the extent of glycosylation. In general, enzymes having high redox-potential are favorable for enzyme catalyzed reactions [246, 248, 249]. Redox-potential of ligninolytic enzymes is as follows: LiP>MnP=VP>laccase (**Table 2.6**).

Table 2.6. Characteristics of extracellular ligninolytic enzymes [250, 251]

Ligninolytic enzymes	Molecular mass (kDa)	Redox potential (mV)	Glycosylation (%)	Isoelectric point	Cofactor
Laccase	50 – 80	0.3 – 0.8	10 – 20 (N-Glycosylated)	3 – 4	O_2
Lignin peroxidase	35 – 48	1 – 1.2	20 – 30 (N-Glycosylated)	3.1 – 4.5	H_2O_2
Manganese peroxidase	38 – 62	0.8 – 1	5 – 15 (N-Glycosylated)	3 – 7.2	H_2O_2
Versatile peroxidase	40 – 47	>1	N.A.	3.4 – 4.9	H_2O_2

"N.A.": not available

Glycosylation, a complex enzymatic process, is responsible for the formation of biopolymers such as polynucleotides at the cellular level [252, 253]. Glycosylation in extracellular enzymes can influence their shape, structure, composition and the formation of substrate binding sites as well as their properties such as redox-potential, enzymatic activity and catalytic potential [254, 255]. Stability of enzymes tends to improve with the increase of glycosylation but it may not always improve the catalytic potential of an enzyme [256, 257]. Deglycosylation of extracellular enzymes has been observed to adversely affect the enzymatic activity, stability and catalytic potential of enzymes [255, 258, 259]. Notably, the catalytic potential or redox-potential of LiP is higher than other ligninolytic enzymes, possibly because the level of glycosylation in LiP is greater than other ligninolytic enzymes [246, 250]. Isoelectric point is important to estimate the charge on fungal enzymes at different pH [260]. Isoelectric point of ligninolytic enzymes mostly falls in acidic pH range *i.e.* 3-7, indicating that ligninolytic enzymes are negatively charged at pH ≥7.0 [261].

2.4.2. Modes of TrOC degradation by WRF

Different WRF and their ligninolytic enzymes have been investigated under different experimental conditions for the removal of TrOCs - either by whole-cell WRF culture or by using the crude and/or purified enzyme extracts. Different modes of TrOC degradation *via* WRF and their ligninolytic enzymes are critically discussed as follows.

2.4.2.1. Removal by Whole-cell WRF

Although whole-cell WRF cultures, either in submerged or solid media, have been used for the removal of TrOCs, submerged whole-cell WRF cultures have been more commonly reported. Since WRF species harbor different enzyme systems, the extent of TrOC removal achieved by different WRF species also varies. TrOC removal performance by different WRF species is presented in **Table 2.7**. Notably, temperature and pH for these studies were in the range of 25-30^{o}C and 4.5-5, respectively, with glucose used as the common electron donor.

T. versicolor, also known as *Coriolus versicolor* [262, 263], has been investigated in several studies for the removal of TrOCs both in batch and continuous-flow bioreactors [234, 237, 264-268]. Depending on the strain, *T. versicolor* may contain laccase, LiP and MnP, with laccase being the predominant enzyme in some strains. It can be observed from **Table 2.7** that *T. versicolor* achieved significant removal (>70%) for most of the tested TrOCs. TrOCs such as steroid hormones, nonylphenol and octocrylene can induce endocrine disrupting effects [269, 270]. Their removal by *T. versicolor* in literature ranges from 80 to 99% regardless of the operating conditions. Conversely, poor and unstable removal was reported for particular pharmaceuticals, namely ciprofloxacine (35%), salicylic acid (0-5%), azithromycin (26%), tetracyclin (0-5%) and carbamazepine (negligible to 90%). Low and/or unstable removal of these compounds can be attributed to the presence of strong electron withdrawing functional groups (EWGs) in their chemical structure [222]. Compared to the pharmaceuticals, the removal of personal care products has been communicated in only a limited number of studies [266, 267]. However, *T. versicolor* has been shown to achieve high removal of ingredients of personal care products such as triclosan, oxybenzone, octocrylene and nonylphenol (**Table 2.7**).

Table 2.7. Removal (%) of TrOCs by different species of WRF (whole-cell) under different operating conditions

WRF Specie	Bioreactor Type	Operating conditions	TrOCs	Initial concentration (mg/L)	Removal efficiency (%)	References
P. chrysosporium (LiP, MnP)	Stirred tank (Batch-fed)	Inoculum= 1.2 g/L Reactor volume = 2 L, HRT= 24 h Mixing speed = 200 rpm pH = 4.5; Temperature= 30ºC Electron donor= glucose Operating time= 30 days	Diclofenac Ibuprofen Naproxen	0.8 0.8 0.8	>99 75-99 >99	[271]
	Stirred tank (Continuous)	Reactor volume = 1.5 L HRT= 24 h pH = 4.5; Temperature= 30ºC Electron donor= glucose Operating time= 50 days	Diclofenac Ibuprofen Naproxen Carbamazepine Diazepam	1 1 1 0.5 0.25-0.5	92 95 95 25-60 0	[272]
T. versicolor (Laccase, LiP, MnP)	Fixed bed (Continuous)	Inoculum= 3.2 g/L Reactor volume = 0.13 L HRT= 120 days pH = 4.5; Temperature= 22ºC Electron donor= glucose Operating time= 26 days	17β-estradiol (E1) 17α-ethynylestradiol	3-18.8 7.3	>95 >95	[264]
	Fluidized bed (Batch-fed)	Inoculum= 3.8 g Reactor volume = 1.5 L pH = 4.5; Temperature= 25ºC Electron donor= glucose Operating time= 15 days	Carbamazepine	0.05-9	61-94	[268]
	Fluidized bed (Batch-fed)	Inoculum= 1.5 g/L Reactor volume = 10 L pH = 4.5; Temperature= 25ºC Electron donor= glucose Operating time= 8 days Real hospital wastewater Non sterile conditions	Ibuprofen Acetaminophen Ketoprofen Ciprofloxacine Azithromycin Propranolol Carbamazepine	2.34 1.56 0.08 84.71 4.31 0.06 0	100 100 100 35 100 100 -50	[237]
	Fluidized bed (Continuous)	Inoculum= 1.4 g/L Reactor volume = 10 L pH = 4.5; Temperature= 25ºC Electron donor= glucose	Acetaminophen Naproxen Ibuprofen Ketoprofen	109 1.62 35.5 2.17	100 100 100 95	[273]

WRF Specie	Bioreactor Type	Operating conditions	TrOCs	Initial concentration (mg/L)	Removal efficiency (%)	References
		Operating time= 8 days Real hospital wastewater Non sterile conditions	Diclofenac	0.477	100	
			Codeine	0.606	100	
			Phenazone	0.497	96	
			Salicylic Acid	0.606	0	
			Ofloxacin	3.34	98	
			Ciprofloxacine	13.0	99	
			Sulfamethoxazole	1.41	100	
			Trimethoprim	0.853	100	
			Metronidazole	0.912	85	
			Azithromycin	1.37	26	
			Clarithromycin	2.20	80	
			Erithromycin	0.008	100	
			Tetracyclin	0.011	0	
			Caffeine	149	39	
			Carbamazepine	0.056	0	
			Atenolol	2.99	75	
			Metoprolol	0.019	95	
	Membrane bioreactor (Continuous)	Inoculum= 3.5 g/L Reactor volume = 5.5 L, HRT= 1 day pH = 5.4; Temperature= 27ºC Operating time= 90 days Non sterile conditions	Diclofenac	0.3-1.5	55	[234]
	Membrane bioreactor (Continuous)	Inoculum= 3 g/L Reactor volume = 5.5 L, HRT= 2 day pH = 4.5; Temperature= 27ºC Operating time= 110 days Non sterile conditions	Ibuprofen	0.005	95	[266]
			Naproxen	0.005	95	
			Ketoprofen	0.005	90	
			Diclofenac	0.005	50	
			Carbamazepine	0.005	20	
			Metronidazole	0.005	40	
			Gemfibrozil	0.005	95	
			Amitriptyline	0.005	85	
			Estriol (E3)	0.005	>95	
			17-β-estradiol	0.005	>95	

WRF Specie	Bioreactor Type	Operating conditions	TrOCs	Initial concentration (mg/L)	Removal efficiency (%)	References
			17-β-estradiol – acetate	0.005	>95	
			17-α ethinylestradiol	0.005	>95	
			Triclosan	0.005	95	
			Benzophenone	0.005	80	
			Oxybenzone	0.005	92	
			Octocrylene	0.005	94	
	Erlenmeyer flask (Batch-fed)	Inoculum= 2-3 mg/test pH = 4.5; Temperature= 28°C Operating time= 14 days Electron donor: glucose	Triclosan	10	98	[267]
			17-α ethinylestradiol	10	94	
			Nonylphenol	10	90	
P. ostreatus (Laccase, MnP)	Erlenmeyer flask (Batch-fed)	Inoculum= 2-3 mg pH = 4.5; Temperature= 28°C Operating time= 14 days Electron donor: glucose	Triclosan	10	98	[267]
			17-α ethinylestradiol	10	62	
			Nonylphenol	10	93	
D. squalens (Laccase, MnP)	Erlenmeyer flask (Batch-fed)	Inoculum= 2-3 mg pH = 4.5; Temperature= 28°C Operating time= 14 days	Triclosan	10	98	[267]
			17-α ethinylestradiol	10	78	
			Nonylphenol	10	85	
B. adusta (Laccase, LiP, MnP)	Erlenmeyer flask (Batch-fed)	Inoculum= 2-3 mg pH = 4.5; Temperature= 28°C Operating time= 14 days	Triclosan	10	98	[267]
			17-α ethinylestradiol	10	78	
			Nonylphenol	10	85	

Other WRF species such as *P. chrysosporium* [271, 272], *B. adusta* , *D. squalens* and *P. ostreatus* containing different combinations of ligninolytic enzymes have also been investigated for the removal of TrOCs (**Table 2.7**). However, these studies reported the removal of only a few TrOCs such as ibuprofen, diclofenac and triclosan. Despite the difference in enzyme secretion pattern of these WRF, efficient removal (in the range of 70 to 99%) was achieved for most tested TrOCs, with uncertain/unstable removal reported for carbamazepine, which is a known persistent pharmaceutical. It is important to note that a direct comparison of TrOC removal data from different studies may not be valid due to the differences in operating conditions and bioreactor configurations as well as difference in their enzymatic systems. However, **Table 2.7** serves the purpose of providing a general overview.

2.4.2.2. WRF bioreactor configurations

Different bioreactor configurations have been explored for the continuous treatment of TrOCs in WRF-based systems (**Table 2.7**). Since whole-cell WRF based treatment systems are still in their development phase, the bioreactor design and configurations are of significant importance. Hence, the salient features of the bioreactor configurations studied to-date for the removal of TrOCs, namely, stirred tank bioreactors [271, 272], bubble column bioreactors, fluidized or packed bed bioreactors [274-276] and membrane bioreactors [277-280] are briefly discussed here to provide a general overview.

Among all the bioreactor configurations, stirred tank bioreactor has been the most common type of bioreactor used for the treatment of TrOCs in WRF based treatment systems. This bioreactor type has been explored mostly in batch and sterile modes [271, 272]. In this bioreactor, aeration is provided usually at the bottom of the bioreactor, which is dispersed *via* mechanical agitation. Mechanical mixing also ensures the uniform mixing of the growth medium and wastewater in the bioreactor [274]. Enhanced production of ligninolytic enzymes could be achieved in stirred tank bioreactors as compared to other bioreactor configurations. For instance, Babič and Pavko [281] investigated the production of laccase and MnP from *D. squalens* in stirred tank bioreactor and bubble column bioreactor under different operating conditions such as incubation time and agitation speed: they observed that laccase production was as much as 70% higher in the former, although the production of MnP was comparable [281]. Agitation speed and high shear rate may influence the morphology of the fungal biomass. In a study by Cao et al. [282], impact of two bioreactor configurations, namely stirred tank bioreactor and airlift bioreactor, on the morphology of *P. sanguineus* was investigated. They found that the morphology of the fungal biomass was adversely impacted in the stirred tank bioreactor, which uses strong mechanical mixing. Consistent with the finding of Cao et al. [282], it has been reported that excessive agitation may lead to the

rupturing of fungal hyphae [274, 283, 284]. Therefore, agitation/mixing speed in bioreactors is an important parameter governing fungal morphology and enzymatic activity.

Fluidized bed bioreactor is another type that has been used for the removal of TrOCs from municipal and synthetic wastewater [237, 273]. In this type of bioreactor, fungal biomass rapidly moves around the solid carrier ('bed'), allowing uniform mixing of the reaction media [285]. However, aggregation of fungal biomass may cause poor fluidization, resulting in spouting of bed. Biomass aggregation can be avoided by intermittent and partial purging of the biomass [286].

A bubble column bioreactor coupled with a microfiltration membrane has been recently explored for the removal of TrOCs [234, 266]. Such fungal membrane bioreactor was expected to offer some additional advantages over bubble column reactors [227, 287, 288] such as: (i) formation of biofilm on the membrane surface that may enhance the removal of recalcitrant TrOCs; (ii) maintenance of high fungal biomass concentration improving biodegradation rate; and (iii) effective prevention of enzyme washout. Nevertheless, bacterial contamination may hamper the growth and enzymatic activity of whole-cell WRF in any bioreactor configuration. Impacts of bacterial contamination on WRF are discussed in the next section.

2.4.2.3. Performance under non-sterile environment and bottlenecks

The capacity of WRF for TrOC removal has been commonly investigated under sterile conditions to avoid bacterial contamination. However, several studies have cast light on the aspect of bacterial contamination by operating bioreactors under non-sterile environment using either synthetic [234, 266] or real wastewater [237, 268, 273, 289-291]. For example, Yang et al. [234] investigated the performance of whole-cell *Trametes versicolor* for the removal of bisphenol A and diclofenac in a membrane bioreactor under non-sterile conditions using a malt-based synthetic wastewater. They observed that the removal of diclofenac was reduced by 40-50% under non-sterile conditions as compared to its 99% removal achieved in sterile batch experiments. In that study, bacterial contamination was evident from microbial analysis. A few recent studies have investigated the removal of pharmaceuticals and endocrine disrupting compounds from municipal and hospital wastewater by whole-cell *Phanerochaete chrysosporium* or *Trametes versicolor* [237, 268, 273, 289-292]. In all these studies, bacterial contamination restricted long term operation of the bioreactors as the overall removal of the TrOCs gradually reduced as compared to that obtained under sterile conditions. Two probable modes of bacterial interruption to fungal enzymatic expression can be perceived: (i) loss of enzyme secretion capacity of fungi owing to the growth disruption under competition for substrate and bacterial colonization of the mycelia, and (ii) destabilization or consumption of secreted enzyme by bacteria [234, 285, 293]. Bacteria are fast growing prokaryotes as compared to eukaryotic WRF and can easily outperform WRF in substrate utilization [293, 294].

In addition to bacteria, other species of fungi can interrupt WRF growth and enzymatic activity. For instance, Badia-Fabregat et al. [291] analyzed the composition of microbial communities in a fluidized bed bioreactor treating hospital wastewater. They observed other fungal species (*e.g. Trichoderma asperellum* and *Trichoderma spp.*) to overtake the originally inoculated fungi (*Trametes versicolor*) in the bioreactor. This is the only study demonstrating the dominance of fungal species other than the inoculated fungi in the bioreactor. Therefore, more research is needed to analyze the presence of different competing species that can suppress the growth of inoculated WRF to formulate strategies to control their proliferation in the bioreactor. A number of strategies for the control of microbial contaminations have been reviewed previously [295]. However, these strategies could only extend the operation of fungal bioreactors without bacterial contamination for a few weeks. Some of the strategies to avoid bacterial contamination are outlined below:

(i) Operation under acidic pH: Optimum pH for the growth and reproduction of fungi ranges from 4.5-5. Conversely, bacteria grow at or near neutral pH. Bacterial growth can be suppressed by maintaining the pH in the range of 4.5-5. However, this is a temporary solution to the problem of bacterial contamination because some bacteria can eventually adapt to acidic environment [293].

(ii) Immobilization or attached growth of fungi: Immobilization of fungal strains onto different carriers under non-sterile conditions shows promising results. For instance, by immobilizing *C. versicolor* onto a plastic support, Hai et al. [287] was able to prevent bacterial contamination for an extended period of time while operating the reactor under non-sterile conditions for the treatment of an azo dye.

(iii) Nitrogen limited feed: Bacterial contamination can be avoided by using a media deficient of nitrogen. However, this strategy can only help during the startup of the bioreactor. Bacterial contamination would occur with the passage of operating time as bacteria would start consuming carbon and nitrogen available in fungal mycelium [293].

(iv) Coupling of bioreactor with micro-screen: Bioreactors can be coupled with a micro-screen which would retain fungal biomass but allow the washout of bacteria with effluent. Moreover, this strategy will benefit from using shorter hydraulic retention times, which will enhance the washout of bacteria from the reactor [296].

(v) Use of disinfecting agents: Inactivation of bacteria without imposing any harmful effects on fungal biomass can be a promising strategy. Depending on the wastewater characteristics, it is important to carefully select the type, dose and contact time of the disinfectant. Disinfection of wastewater using ozone has been used successfully to selectively inactivate bacteria [297, 298].

(vi) Biomass replacement: Periodic biomass replacement and purging strategy can be used to carry out long term operation of fungal bioreactors. In this strategy, biomass in the bioreactor is purged and renewed in different fractions (*e.g.*, ½ or ¼th of the initial biomass volume) at different frequencies [292, 299].

(vii) Pretreatment of wastewater: Coagulation-flocculation pretreatment of non-sterile wastewater can help to reduce the initial bacterial count which would allow an extended operation of the fungal bioreactor [292].

2.4.2.4. Removal by crude ligninolytic enzymes

Individual extracellular ligninolytic enzyme has been tested for the removal of a wide range of pollutants. Use of the harvested enzyme instead of a whole-cell preparation allows decoupling of fungal growth and pollutant degradation steps, and this can be a suitable strategy to avoid bacterial contamination issues. The capacity of both crude and purified/commercially available extracellular enzymes for TrOC removal has been reported previously [229, 266, 300-307]. However, whole-cell fungi may achieve relatively better removal of a broader spectrum of pollutants than extracellular enzymes due to the availability of extracellular, intracellular and/or mycelium bound enzymes in addition to sorption of pollutants onto fungal biomass.

Crude extracellular enzymes have been investigated for the degradation of TrOCs in both batch and continuous-flow mode. For instance, Wen et al. [308] achieved significant degradation of two pharmaceuticals, oxytetracycline (84%) and tetracycline (72%), by using crude MnP (40 U/L) extracted from *P. chrysosporium*. Similarly, crude enzyme solution extracted from *T. versicolor* containing MnP (30 U/L) and laccase (1500 U/L) was tested for the degradation of 10 pharmaceuticals at an initial concentration of 10 µg/L each [301]. They achieved complete degradation of five pharmaceuticals, *viz* diclofenac, ibuprofen, naproxen, indomethacin and fenoprofen, while the rest were partially removed. LiP extracted from *P. chrysosporium* was tested for the degradation of diclofenac and carbamazepine at different pH [309]. It was observed that degradation of carbamazepine was mostly less than 10%, while a complete degradation was achieved for diclofenac at pH 3-4.5. Similarly, crude extract from *T. versicolor* was used for the removal of 30 TrOCs [302]. The results revealed that all steroid hormones were almost completely removed (>95%), while removal of two TrOCs namely diclofenac and triclosan ranged from 50 to 60% with poor removal of the remaining TrOCs (<20%). In another study [310], crude enzyme extract from *P. ostreatus* achieved low removal (<20 %) for two TrOCs, namely oxybenzone and naproxen.

Since WRF also secretes natural mediators along with extracellular enzymes, crude enzyme may achieve better removal as compared to purified and/or commercially available enzymes. For instance, Tran et al. [301] highlighted that complete removal (>99%) of some pharmaceuticals

such as diclofenac, indomethacin and ibuprofen was due to the presence of some natural mediators in crude laccase solution. Removal of selected TrOCs by individual crude enzymes has been systematically presented in **Table 2.8**. It can be seen that the extent of TrOC removal is different for each type of extracellular enzyme. Moreover, crude enzyme extract from different fungi may perform differently. For instance, Weng et al. [311] collected crude LiP from two WRF species, namely, *P. sordida* and *P. chrysosporium* for the treatment of EDCs and found that LiP from *P. sordida* was more effective than LiP from *P. chrysosporium*. Similarly, removal of DEET, an insect repellent, by *T. versicolor* was 55% in real wastewater as compared to its 20% removal in acetate buffer. High removal of DEET in real wastewater was attributed to the presence of other compounds (such as phenolic compounds) that may act as redox-mediators [312], as further discussed in **Section 2.4.5**.

2.4.2.5. Removal by purified ligninolytic enzymes

Purified or commercially available extracellular ligninolytic enzymes, mostly laccase from different WRF, has been used for the treatment of TrOCs in both batch and continuous bioreactors. Average removal of the selected TrOCs by purified laccase reported in recent studies is presented in **Figure 2.14**. Purified laccases are more effective for the removal of phenolic compounds such as oxybenzone, triclosan and steroid hormones. Removal of non-phenolic compounds such as carbamazepine, naproxen and ketoprofen is generally poor/unstable. Their degradation depends on their physicochemical properties such as hydrophobicity and chemical structure as well as the oxidation reduction potential (ORP) of the enzyme [222, 313]. Indeed, based on the results of the recent studies incorporated in **Figure 2.14**, removal of phenolic compounds ranges from 70 to 99%, while the removal of non-phenolics is generally less than 20%. However, relatively higher removal of some non-phenolic compounds, namely, diclofenac (40-50%), octocrylene (>80%) and ibuprofen (30-45%) has been reported because these compounds contain both electron donating and electron withdrawing functional groups. Although both crude and purified enzymes demonstrated degradation of a range of pollutants, crude enzymes achieved better removal of some TrOCs such as diclofenac and naproxen as compared to purified enzymes [229, 266, 300, 302, 303, 306], possibly due to the presence of natural mediators in crude enzyme solution secreted by WRF. For instance, Tran et al. [301] achieved almost complete removal of three pharmaceuticals namely diclofenac, ibuprofen and naproxen after treatment with crude enzyme extracted from *T. versicolor*, whereas purified laccase from *T. versicolor* and *A. oryzae* achieved 20-50% removal of these compounds [229, 303, 304, 314].

Table 2.8. Performance of crude enzymes for the removal (%) of the selected TrOCs

TrOCs	**Laccase** [231, 301, 302, 310, 312, 315-321]			**LiP** [309, 311, 322]			**MnP** [301, 317, 318, 323]		
Non-phenolic compounds	Removal (%)	Initial concentration (mg/L)	Incubation time (h)	Removal (%)	Initial concentration (mg/L)	Incubation time (h)	Removal (%)	Initial concentration (mg/L)	Incubation time (h)
Carbamazepine	5 – 37	0.01-0.1	24-48	<10	5	2	14-20	0.01-4.7	24-48
Ibuprofen	<5 – 38	0.01-0.1	24-48	-	-	-	20	0.01	48
Naproxen	20-100	0.01-0.5	24-48	-	-	-	95	0.01	48
Diclofenac	60-100	0.01-0.1	3-48	>99	5	2	100	0.01	48
Gemfibrozil	20-25	0.01-0.1	24-48	-	-	-	30	0.01	48
ketoprofen	<5 -12	0.01-0.1	24-48	-	-	-	22	0.01	48
Clofebric acid	10-20	0.01-0.1	24-48	-	-	-	<10	0.01	48
Benzophenone	<5	0.1	24	-	-	-	-	-	-
DEET	20-55	0.01	4	-	-	-	-	-	-
Octocrylene	20	0.1	24	-	-	-	-	-	-
Phenolic compounds									
Estrone	70-100	0.1-27	1-24	60	27	24	>99	5	8
17β-Estradiol	>99	0.01-5	1-24	40, 85	0.6, 27	1, 24	>99	2.96	1
17α-Ethynylestradiol	>99	0.1-2.96	1-24	20, 82	6.6, 30	8, 24	>99	2.96	1
Oxybenzone	10-25	0.1-0.5	24	-	-	-	-	-	-
Nonylphenol	100	22	1	-	-	-	-	-	-
Triclosan	70-90	0.1-144	24	-	-	-	-	-	-

"-": not reported

Utilization of crude enzymes for the treatment of TrOCs may considerably reduce the cost of the treatment process. However, extracellular extract *i.e.*, the crude enzyme solution also contains significant amount of the unspent growth media, and dosing crude enzyme means additional organic loading from this.

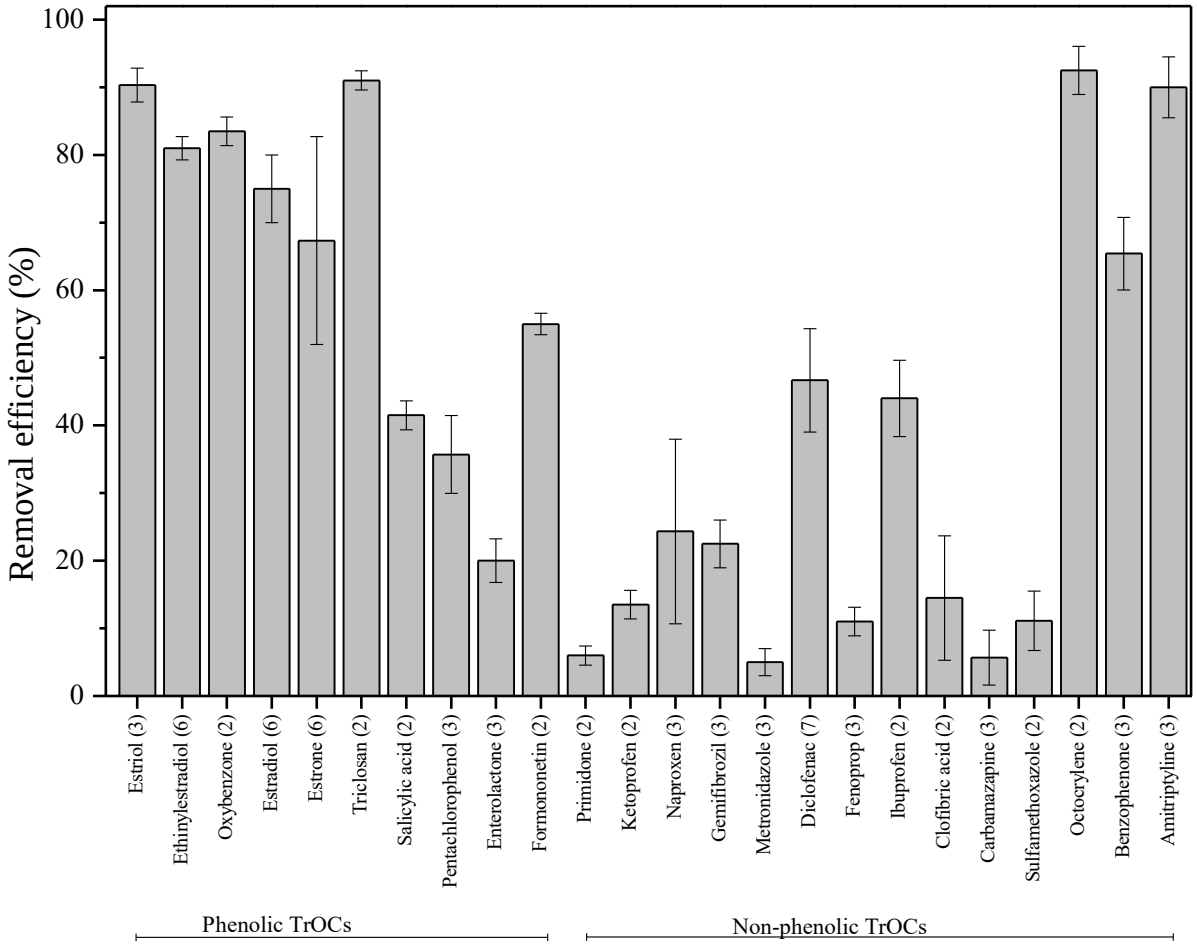

Figure 2.14. Average removal of phenolic and non-phenolic TrOCs after treatment with purified/commercially available laccase. Error bar indicates average±standard deviation. Numbers within parenthesis indicates number of data points. Data was collected from the following studies: [229, 303, 304, 306, 314, 324-328].

2.4.2.6. Mechanisms of TrOC removal

Removal mechanisms during treatment with WRF whole-culture include: (i) sorption onto the fungal biomass; (ii) degradation by extracellular enzymes; and (iii) degradation by mycelium bound or intercellular enzymes. A schematic of fungal mediated treatment process with possible removal mechanisms is presented as **Figure 2.15**. Hydrophobicity (log D) of TrOCs is a key property that governs biosorption onto fungal biomass and could facilitate enhanced removal of some compounds. For instance, a batch study to investigate the contribution of biosorption and

degradation by extracellular enzymes confirmed that hydrophobic TrOCs (log D>4) were highly removed by both mechanisms [300]. Moreover, they also confirmed that biosorption of significantly hydrophobic compounds facilitated the biodegradation of these compounds. On the other hand, a few studies have reported that removal of some TrOCs such as 17β-Estradiol, 17α-ethynylestradiol, triclosan and nonylphenol by whole-cell WRF and extracellular enzymes is comparable (*see* **Table 2.7 and 2.8**), indicating a negligible impact of biosorption on their removal.

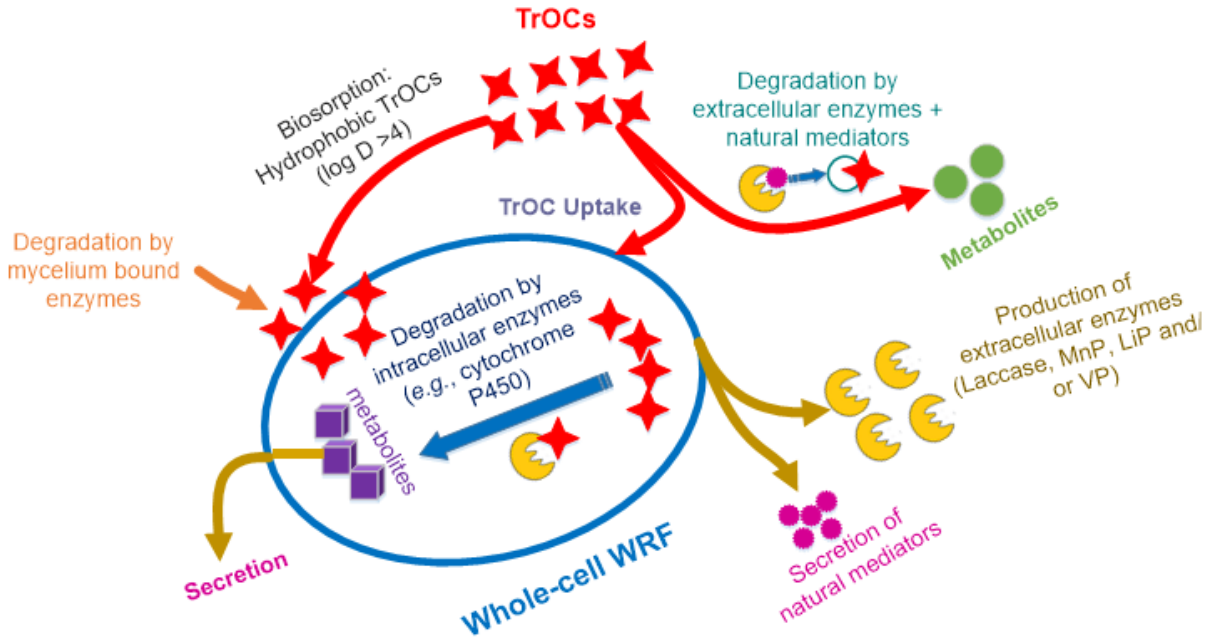

Figure 2.15. TrOC removal mechanisms by WRF-based treatment processes. Adapted from [329].

Biodegradation by whole-cell can be due to intracellular, extracellular and mycelium-associated enzymes. This can lead to significant differences in removal by whole-cell WRF and harvested enzyme. For instance, carbamazepine, containing a strong EWG, was significantly removed by some WRF species, namely *P. ostreatus* (100%) [247] and *T. versicolor* ATCC 42530 (76%) [301], while crude [247, 301, 302] and purified laccase [229, 306] could only achieve 5-15% carbamazepine removal. Similarly, ibuprofen and naproxen were almost completely removed by whole-cell WRF [237, 265, 273]. In contrast, their removal by crude and purified laccase was in the range of 10 to 40% [229, 266, 306, 314]. Since both naproxen and ibuprofen are hydrophilic compounds (log D <3), role of biosorption in their removal would be limited. However, almost complete removal of these compounds by whole-cell WRF substantiates the role of mycelium bound and/or or intercellular enzymes. Indeed, the role of intercellular enzyme (*i.e.*, cytochrome P450) in the degradation of naproxen, diclofenac and carbamazepine has been demonstrated [247, 265, 330]. These studies showed that naproxen, diclofenac and carbamazepine were partially removal (15-50%) in the presence of cytochrome P450 inhibitor during whole-cell WRF treatment.

Therefore, it can be concluded that high removal of some TrOCs in whole-cell WRF treatment is due to the synergetic effects of extracellular, intercellular and/or mycelium bound enzymes. Moreover, secretion of natural mediators may also help in enhancing the removal of these compounds.

2.4.2.7. Degradation pathways, identification of intermediates and toxicity

TrOCs degradation pathways and their intermediate products have been identified for some compounds such as carbamazepine, diclofenac, triclosan and ibuprofen. However, each WRF species may follow a different degradation pathway.

Fungal mediated treatment of diclofenac starts with the conversion of the hydroxyl group in its structure into an intermediate product, namely, hydroxy diclofenac. Hydroxy diclofenac can be amenable to further fungal biodegradation [330, 331]. In *vivo* and in *vitro* experiments for the degradation of diclofenac showed that laccase (*T. versicolor*)-catalyzed degradation leads to the formation of biodegradable compounds such as: (i) hydroxelated metabolites (appeared in both *in vivo* and *in vitro* experiments); and (ii) 4-(2,6 dichlorophenylamino)-1,3-benzenedimethanol metabolite (appeared only in *in vivo* experiments) [331]. Diclofenac and its metabolites both disappeared after 24 h of incubation, reducing the ecotoxicity of the treated effluent. Degradation of ketoprofen was reported to initiate by the intercellular cytochrome P450 that converts ketoprofen into (2-[3-(4-hydroxybenzoyl)phenyl]-propanoic acid) and (2-[(3-hydroxy(phenyl)-methyl)phenyl]-propanoic acid) *via* hydroxylation and reduction of the ketone group, respectively [331]. Moreover, it was also observed that the role of extracellular enzyme (laccase) in the degradation of ketoprofen was insignificant.

Both laccase and cytochrome P450 can catalyze the degradation of naproxen in WRF based treatment. Formation of two intermediates, namely 6-desmethylnaproxen and 1-(6-methoxynaphthalen-2-yl)ethanone), was observed possibly *via* P450-mediated desmethylation and laccase catalysis, respectively [265, 272]. Moreover, naproxen and associated intermediates were completely removed from the reaction media and the treated effluent was non-toxic [265].

Ibuprofen conversion predominantly starts with the formation of hydroxy-ibuprofen *via* hydroxylation. Marco-Urrea et al. [332] investigated the degradation pathways of ibuprofen by WRF *T. versicolor*. They observed that oxidation of isopropyl chain resulted in the formation of 1-hydroxy ibuprofen and 2-hydroxy ibuprofen. These intermediates were then degraded by *T. versicolor* to 1,2-dihydroxy ibuprofen, which was not further degraded. Hence, the ecotoxicity of treated effluent was higher than that of the initial solution [332].

Degradation pathways for carbamazepine by different whole-cell WRF including *T. versicolor* and *P. ostreatus* have also been studied. These have identified several stable intermediate products

namely, 10,11-dihydro-10,11-dihydroxy-carbamazepine, acridine, acridone and 10,11-epoxy-carbamazepine. Interestingly, the treated effluent showed less toxicity [247, 268].

Intermediates or metabolites of triclosan during whole-cell treatment depends on WRF species. Major intermediates of triclosan during the treatment with *C. polyzona* WRF were dimer, trimer and tetramer [333], while 2-O-(2,4,4-trichlorodiphenyl ether)-b-D-xylopyranoside, 2-O-(2,4,4-trichlorodiphenyl ether)-b-D-glucopyranoside and 2,4-dichlorophenol were identified as major intermediates following its treatment with *T. versicolor* [334].

2.4.3. Impacts of physicochemical characteristics of wastewater on TrOC removal

Performance of WRF in wastewater treatment depends on several factors such as environmental conditions and physiochemical properties of the wastewater as well as the properties of TrOCs. Influence of TrOC properties on their overall removal in a WRF mediated treatment process has been comprehensively reviewed previously by Asif et al. [329]. Briefly, physicochemical properties of water/wastewater such as pH, temperature and the presence of dissolved organic and/or inorganic compounds may influence the performance of both WRF and their ligninolytic enzymes. Because information about these properties are vital to design and optimize WRF- and enzyme-based treatment systems, these are discussed in this section.

The operating temperature not only affects the stability of WRF/enzymatic systems but also the rate of reaction. It is believed that the rate of reaction increases with increase in temperature [309]. However, depending on the WRF strain, rapid thermal inactivation of ligninolytic enzymes has been observed at temperature above 40^{o}C [335, 336]. Only a few studies have investigated the impact of temperature on enzymatic activity and removal efficiency in a reaction media. For instance, increase in temperature of the reaction media from 20 to 25^{o}C enhanced the removal efficiency of chlorophenols in laccase-mediated treatment system [337]. Similarly, Ullah et al. [338] investigated the removal of pentachlorophenol by varying the temperature from 10 to 45 ^{o}C and found that the optimal temperature was 25^{o}C to achieve maximum laccase activity and pentachlorophenol removal. Temperature range of 37-40 ^{o}C has been reported to achieve optimal activity of MnP and LiP [308, 339].

The pH for optimum activity can vary depending on the source of enzyme. For instance, laccases extracted from *Pleurotus ostreatus* [340], *Trametes versicolor* [341], and *Albatrella dispansus* [342] have been reported to show maximum laccase activity at a temperature of 35, 50 and 70 ^{o}C, respectively. However, in general, the optimum temperature for most fungal laccases and peroxidases ranges from 25-30^{o}C and 35-40^{o}C, respectively [308, 337, 339]. Depending on the source fungus, the optimum pH for high and stable laccase activity ranges from 3.5 – 6.0 [343]. For example, the optimum pH for activity of laccase from *Trametes versicolor* [344, 345],

Physisporinus rivulosus [346] and *Agaricus blazei* [347] was 3.0-4.5, 4.0 and 5.5, respectively. Best removal of TrOCs ubiquitously detected in wastewater such as triclosan, diclofenac, ketoprofen and bisphenol A was achieved at pH range of 4.0-6.0 [233, 303, 311, 315, 348, 349]. The optimum pH varies for different types of TrOCs due to the difference between the redox-potential of the TrOC and enzymes [350, 351]. In general, removal of TrOCs at varying pH results in a bell-shaped curve because TrOC removal reduces with the increase in the pH of the reaction mixture [303, 348]. Reduction in the removal of TrOCs with the increase in pH can be attributed to: (i) the change in the redox-potential of enzymatic reactions; and (ii) the binding of hydroxide ions to Type II and Type III copper sites of laccase at alkaline pH, thereby blocking the internal electron transfer [352, 353].

Fungal/enzymatic bioreactors have mostly been studied for the treatment of synthetic wastewater spiked with TrOCs in absence of potential inhibiting compounds prevalent in real wastewater [222, 324]. However, wastewater derived interfering compounds can affect the stability and catalytic efficiency of WRF and ligninolytic enzymes [354, 355]. Many compounds such as sulphides, halides [350, 356], natural/synthetic organics [357-362] and heavy metals [231, 363] can inhibit the catalytic activity of laccases [364]. Moreover, each compound may have different mode of laccase inhibition. For instance, fatty acids inhibit laccase catalytic potential by blocking the binding sites for phenolic substrates [365-367]. On the other hand, spectrophotometric assays, electron spin resonance spectroscopy and catalytic voltammetry analysis confirmed that anionic inhibitors such as halides and sulphides could block the access to the active copper sites in laccase [368-370]. Among anionic inhibitors, fluoride and azide are the most effective and can rapidly reduce the catalytic activity of laccase by 50% even at µM concentrations [371]. Although inhibition of laccases by halides can proceed in the following order: fluoride > chloride > bromide, the concentration of halides required to inhibit laccases varies, with no fixed correlation with their inhibition potential. For instance, chloride concentration ranging from 100 µM – 100 mM may cause a 50% drop in activity of laccase from different species [242, 372].

2.4.4. TrOC removal by enzymatic membrane bioreactor (EMBR)

Enzyme washout had been the major limitation of enzyme applications in continuous-flow reactors. These limitation can be solved by immobilizing enzyme onto a support material [319, 373] or by coupling the enzymatic bioreactor with a membrane [229, 374]. Compared to enzyme immobilization, an EMBR offers several benefits over immobilized enzyme including ease of operation and maintenance as well as limited mass transfer limitation.

Lloret et al. [243] investigated the removal of two estrogens (estrone and 17β-estradiol) by using a suspension of a commercially available laccase from *M. thermophila* in the EMBR. The UF membrane (MWCO of 10 kDa), which was submerged in the enzymatic bioreactor, effectively

retained the enzyme. The EMBR effectively removed to (64 – 100%) estrone and 17β-estradiol at an enzymatic activity of 500 U/L. In addition to the high removal efficiency, the EMBR effectively reduced estrogenic activity to 97%. Similarly, Nguyen et al. [229] investigated the removal of 30 TrOCs in UF-EMBR using purified laccase from *A. oryzae*. They observed that the removal of phenolic TrOCs with EDGs ranged from 70 to 99%, while the removal of phenolic TrOCs with EWGs was in the range of 25 to 50%. On the other hand, removal of tested non-phenolic compounds were generally poor (*i.e.* 5-20%) expect for a few non-phenolic TrOCs namely, benzophenone (75%), amitriptyline (95%), octocrylene (99%) and diclofenac (45%) [229]. A list of EMBR studies for the removal of TrOCs is presented in **Table 2.9**.

Denaturation of enzyme (*i.e.*, loss of enzymatic activity) has been observed during the operation of EMBR. Denaturation of enzyme may be due to various factors including physicochemical and biological inhibitors. Denaturation of enzyme can lead to the reduction in the removal of TrOCs by the EMBR system. Reduction in enzymatic activity has been observed in several studies [229, 303, 375]. Thus, periodic enzyme addition may be necessary to maintain stable enzymatic activity and TrOC removal. Hata et al. [375] re-injected laccase every 8 h to the bioreactor to enhance the performance of the laccase-catalyzed treatment system. Similarly, Nguyen et al. [229] re-injected laccase every 12 h to maintain laccase activity in an UF-EMBR.

Table 2.9. Details of the selected studies for the removal of TrOCs by EMBR

Membrane type (MWCO)	Laccase (μM/min)	Reactor volume (L)	HRT (h)	pH	Temperature (ºC)	Wastewater composition	References
UF Polyethersulfone (10 kDa)	500, 100	0.25	1, 4	7	26	TrOCs (4 mg/L each) in water buffered with 100 mM sodium phosphate	[243]
UF Polyethersulfone (10 kDa)	500	0.25	2, 4	4, 7	25	TrOCs (4 mg/L each) in water buffered with 100 mM sodium acetate at pH 4 and with 100 mM sodium phosphate at pH 7	[376]
UF Polyacrylonitrile (6 kDa)	90	1.5	8	6.8	28	TrOCs (0.5 mg/L each) in Milli-Q water	[303]
UF Polyacrylonitrile (6 kDa)	180	1.5	8	6.8	28	TrOCs (0.005 mg/L each) in Milli-Q water	[229]
UF Polyacrylonitrile (6 kDa)	90	1.5	8	6.8	28	TrOCs (0.25 and 0.5 mg/L each) in Milli-Q water	[306]
Laccase-grafted ceramic membrane (0.2 and 1.4 μm)	NA	2	24	7	25	TrOCs (20 mg/L each) in deionized water	[377]
Laccase on TiO_2 coated on PVDF membrane	0.42	0.04	24	5.5	22	TrOCs (35 mg/L each) in water buffered with 100 mM acetate	[378]

"NA": not available

Interestingly, during the operation of UF-EMBR, removal of some TrOCs such as naproxen, oxybenzone and pentachlorophenol was better as compared to batch experiments as explained here [306, 374]. Although a direct comparison between batch and continuous experiments may not be valid, TrOC removal data obtained from batch enzymatic bioreactor and UF-EMBR (**Figure 2.16**) indicates two distinct pattern: (i) significant improvement (40-90%) in the removal of some phenolic TrOCs such as amitriptyline, benzophenone and octocrylene and some phenolic TrOCs such as oxybenzone, pentachlorophenol, and salicylic acid during the operation of UF-EMBR, and (ii) 5-20% reduction in the removal of some phenolic compounds such as estrone, 17β – estradiol and bisphenol A.

Improvement in the removal of some non-phenolic and phenolic compounds was attributed to retention of these compounds on enzyme gel-layers formed on the surface of the membrane during EMBR operation [229]. Since laccase is negatively charged at pH ≥6, formation of an enzyme gel-layer may have converted the membrane surface from an uncharged to a partially charged membrane. Although ultrafiltration membrane used by Nguyen et al. [229] was not expected to retain TrOCs *via* size exclusion mechanism, enhanced removal of some TrOCs can be attributed to: (i) adsorption of hydrophobic TrOCs (log D>3) onto enzyme gel-layers; and (ii) non-hydrophobic interaction (*e.g.*, electrostatic interaction) between ionizable TrOCs and negatively charged membrane surface. For instance, removal of the significantly hydrophobic nonphenolic TrOCs such as benzophenone (log D=3.46), octocrylene (log D=5.18) and oxybenzone (log D=3.21) in EMBR improved, possibly due to their adsorption onto enzyme gel-layer (**Figure 2.16**). However, significantly hydrophobic solutes adsorbed on membrane surface could diffuse in permeate to attain the equilibrium. This phenomenon has commonly been reported for nanofiltration and forward osmosis membranes [379, 380] but could be possible in ultrafiltration membranes as well [381, 382]. The evidence (**Figure 2.16**) suggests that diffusion in to permeate can be the reason of reduction in the removal of a few hydrophobic phenolic TrOCs such as estrone, 17β – estradiol and bisphenol A. Moreover, reduction in their removal can also be attributed to the continuous loading of TrOCs in EMBR [229, 243].

Electrostatic interactions between charged membrane and ionizable TrOCs are strongly influenced by solution pH and dissociation constant (pK_a) of compounds [383]. For instance, Nghiem et al. [167, 384] observed that the rejection rate of hydrophilic pharmaceuticals (log <3) *via* charge repulsion by a negatively charged loose NF membrane was increased at pH>pk_a due to the transformation of compounds from neutral to negatively charged species. Since EMBR was operated near neutral pH *i.e.*, 6.8-7 [229, 306], removal of hydrophilic compounds such as ibuprofen (pK_a=4.41), naproxen (pK_a =4.84), ketoprofen (pK_a =4.23) was possibly improved due to their conversion from neutral to negatively charged species. It was also confirmed that the retained TrOCs were subsequently degraded in EMBR [229], indicating that retention of both enzyme and TrOCs is important to enhance the removal of TrOCs in EMBR.

Despite all the possible explanations, change in membrane properties and impacts on TrOC removal due to the formation of enzyme gel layer need focused and thorough investigations.

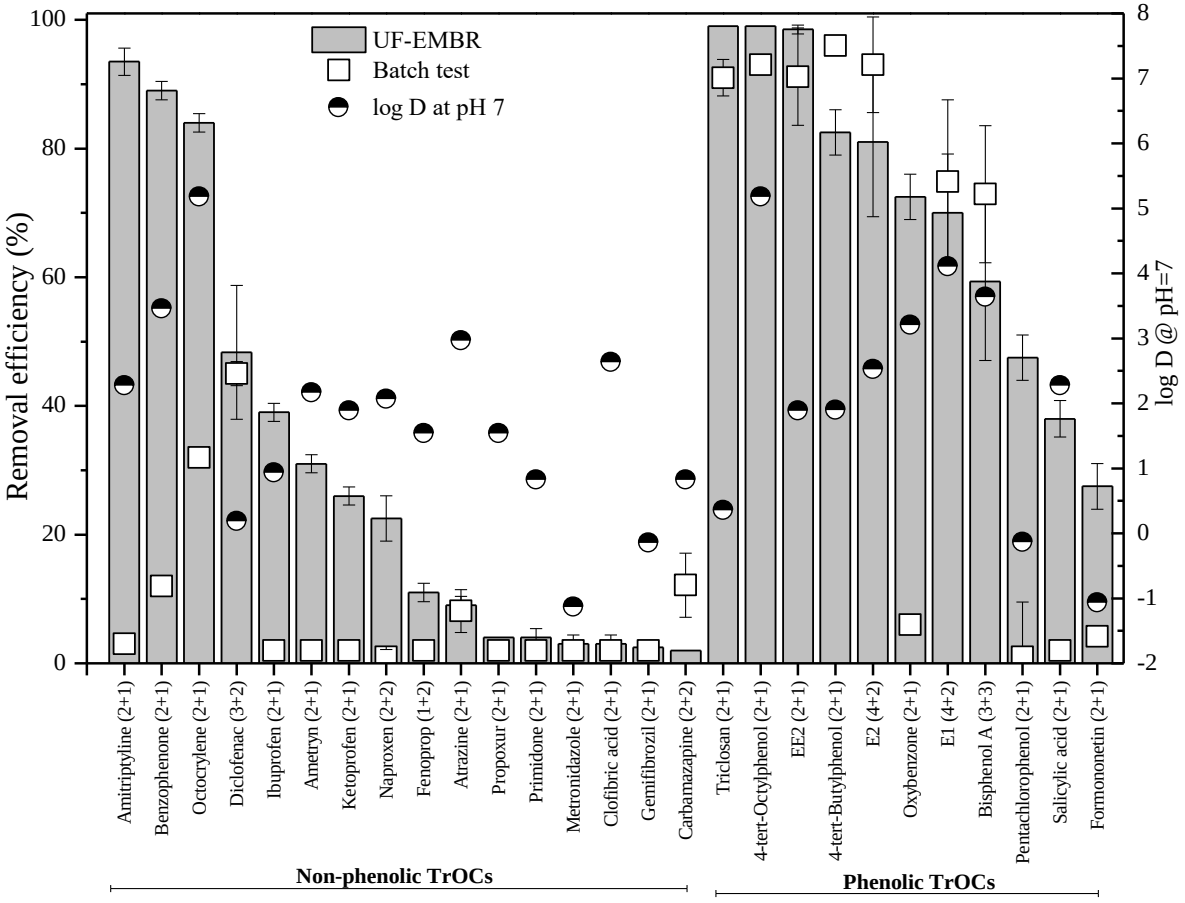

Figure 2.16. Removal of TrOCs in batch enzymatic bioreactor and continuous-flow EMBR. Numbers within parenthesis indicates number of data point (n_{EMBR} +$n_{batch\ laccase}$). Error bars represent standard deviation among data points. E1: Estrone; E2: 17β – Estradiol; EE2: 17α – ethinylestradiol. The data is extracted from the studies listed in **Table 2.9**.

As noted in **section 2.3**, activated sludge-based HR-MBR provides excellent removal of TrOC based on the effluent quality, however the biodegradation of TrOC in the bioreactor is comparable to that achieved by CAS process and/or MBR, leading to the accumulation of TrOCs in the bioreactor of HR-MBR. Therefore, WRF or their ligninolytic enzymes having stronger biodegradation capacity than the activated sludge should be investigated for the biodegradation of TrOCs. Based on the impact of membrane retention in conventional UF-EMBR, it can be postulated that that the coupling of an enzymatic bioreactor with a high retention membrane process may facilitate the degradation of recalcitrant compounds by retaining both enzyme and TrOCs.

2.4.5. Improvement in the performance of EMBR with redox-mediators

Laccase catalyzes mono-electronic oxidation of TrOCs. However, the extent of removal depends on the ORP of the enzyme and individual TrOCs. Poor removal of non-phenolic

TrOCs can be attributed to: (i) presence of a strong EWG in the structure of non-phenolic compounds, causing steric hindrance; and (ii) higher ORP of non-phenolic compounds than laccase [313]. Removal of non-phenolic compounds can be enhanced by introducing a low-molecular weight redox-mediator. In a redox-mediator catalyzed system, highly reactive radicals are formed due to the oxidation of a mediator by laccase, and these radicals then serve as an electron transfer shuttle between TrOCs and laccase, facilitating enhanced removal of recalcitrant compounds. Three oxidation mechanisms, namely hydrogen atom transfer (HAT), ionic mechanisms and electron transfer have been reported for mediators. For instance, 1-hydroxybenzotriazole (HBT) and syringaldehyde (SA) follow HAT mechanism, while 2,2-azino-bis(3-ethylbenzothiazoline)-6-sulfnoic acid (ABTS) and 2,2,6,6-tetramethylpiperidinyloxyl (TEMPO) follow electron transfer and ionic mechanisms, respectively [310, 385].

Properties of different redox-mediators used to enhance the removal of different TrOCs are summarized in **Table 2.10**. Although the mediators perform differently, two mediators namely HBT and SA have been commonly used to broaden the spectrum of compounds significantly degraded by laccase. Studies involving these mediators confirmed that they not only improved the ORP of reaction media but also the extent of removal. Only a few studies elucidated the performance of different mediators based on the type of substrate (*i.e.*, phenolic *vs*. non-phenolic). For instance, N=OH type mediators (VA and HBT) were found to achieve the best removal of non-phenolic TrOCs such as clofibric acid, naproxen and carbamazepine, while SA and ABTS performed better for phenolic compounds such as salicylic acid and steroid hormones [229, 303, 306].

Table 2.10. Properties of redox-mediators used to improve the performance of laccase-based treatment of TrOCs. Adapted from [295].

Redox-mediator	Type of mediator	Free radicals	Oxidation mechanism	Application for TrOC removal
1-hydroxibenzotriazole (HBT)	N-OH/ synthetic	aminoxyl	HAT	PPCPs, EDCs, pesticides and industrial chemicals
Violuric acid (VA)	N-OH/ natural	aminoxyl	HAT	PPCPs, pesticides and industrial chemicals
N-hydroxyphthalimide (HPI)	N-OH/ synthetic	aminoxyl	HAT	PPCPs and pesticides
Syringaldehyde (SA)	$C_6H_4(OH)(OCH_3)$/ natural	phenoxyl	HAT	PPCPs, EDCs, pesticides and industrial chemicals
Vanillin (VAN)	$C_6H_4(OH)(OCH_3)$/ synthetic	phenoxyl	HAT	PPCPs, EDCs, pesticides and industrial chemicals
2,2,6,6-tetramethylpiperidinyloxyl (TEMPO)	N – O / synthetic	Oxoammonium	Ionic	PPCPs, EDCs, pesticides and industrial chemicals

Redox-mediator	Type of mediator	Free radicals	Oxidation mechanism	Application for TrOC removal
2,2-azino-bis(3-ethylbenzothiazoline-6-sulfnoic acid (ABTS)	ABTS/ synthetic	$ABTS^{+}$ and $ABTS^{++}$	Electron transfer	PPCPs, EDCs, pesticides and industrial chemicals

Mediator type, concentration and compound properties influence the performance of a redox-mediator. For instance, removal of diclofenac improved from 40 to 80% by increasing the concentration of SA from 0.1 to 0.5 mM [314]. Similarly, Nguyen et al. [306] achieved an improvement of 35% in the removal of diclofenac in an EMBR by increasing the dose of SA from 0.01 mM to 0.1 mM. However, beyond a threshold concentration increasing redox-mediator dose may not improve the removal of TrOCs. For instance, Ashe et al. [310] observed that increasing the concentration of VA and ABTS from 0.5 to 1 mM could not enhance the removal of oxybenzone and naproxen. They also observed that removal of atrazine was reduced by 15-25 % when the concentration of VA and HBT was increased from 0.1 to 0.25 mM. This may be attributed to the complex interactions between laccase and the radicals generated due to degradation of the mediator by laccase, as discussed below.

It has been observed in almost all the studies that enzymatic activity significantly drops with the addition of mediators. For instance, Hata et al. [375] observed 90% decline in enzymatic activity within fist 8 h of incubation in the presence of HBT. Similarly, rapid decline in enzymatic activity was also observed with the addition of VA, HBT or ABTS [310]. Rate of enzyme inactivation depends on the relative stability of the radicals generated by mediators. In the absence of any known enzyme inhibitor, rapid enzyme inactivation in mediator catalyzed system can be attributed to: (i) the blockage of active sites of the enzyme by charged radicals and metabolites; and (ii) the reaction of metabolites with enzyme-active sites to convert them into non-productive complexes [386, 387].

Despite rapid inactivation of enzymes, redox-mediators can compensate by enhancing the rate of reaction, eventually achieving rapid and enhanced removal of TrOCs. However, periodic replenishment of enzyme is needed to maintain the removal efficiency of TrOCs, constraining the long-term operation of mediator-enzyme based wastewater treatment processes. Therefore, selection of mediator type and its concentration is vital for effective and long-term operation of such systems.

2.5. Performance of emerging advanced oxidation process

In addition to exploring the efficiency of enzymatic degradation, this thesis has explored combined application of enzymatic and emerging advanced oxidation processes (AOPs). In line with this, performance of emerging AOPs such as persulfate (PS) oxidation process is reviewed and is briefly presented in this section.

Due to their molecular properties, conventional biological processes are not effective for a wide range of TrOCs (*see* **Section 2.3**). On the other hand, despite the effective removal of TrOCs

by NF/RO membrane filtration, an additional step is required for the treatment of the produced concentrate. In this context, it is noteworthy that advanced oxidation processes (AOP) may achieve effective degradation of TrOCs, and may degrade pollutants causing membrane fouling. Notably, post treatment of biologically treated wastewater by AOPs may simultaneously achieve disinfection and TrOC removal [388]. Among the advanced oxidation processes, ozonation has been mostly investigated for TrOC removal [389, 390]. However, ozone residuals may interact with membrane material, and can reduce the membrane lifetime [391]. Activated PS is an emerging advanced oxidation process that can degrade both natural organic matter and recalcitrant TrOCs [392, 393].

PS is stable at room temperature, but can be activated by various agents such as transition metals (*e.g.*, iron), heat, and ultraviolet (UV) light to form one or more sulphate radicals ($SO_4^{-\bullet}$), which are highly reactive [392]. PS activation by heat and UV light produce two $SO_4^{-\bullet}$ radicals (Equation 1), while only one $SO_4^{-\bullet}$ radical is generated following activation by transition metals such as Fe^{2+} (Equation 2). This indicates that activation by heat or UV light may provide more efficient treatment compared to activation by a transition metal [392, 394].

$$S_2O_8{}^{2-} + heat\ or\ UV\ light \rightarrow 2\,SO_4{}^{2-\cdot} \qquad \text{Eq. (1)}$$

$$S_2O_8{}^{2-} + Fe^{+2} \rightarrow Fe^{+3} + SO_4{}^{2-\cdot} \qquad \text{Eq. (2)}$$

Depending on wastewater characteristics, persulfate or $SO_4^{-\bullet}$ radicals may react with water and/or organics to form secondary radicals that can also contribute to degradation of organic impurities [392, 395]. $SO_4^{-\bullet}$ radicals can react with water to form hydroxyl ($OH^{-\bullet}$) radicals, but the abundance of the $SO_4^{-\bullet}$ and $OH^{-\bullet}$ radicals is governed by the pH of reaction media. Under acidic conditions (pH<7), $SO_4^{-\bullet}$ radicals are the dominant species, while $OH^{-\bullet}$ is the primary reactive species under basic conditions (pH>7). At neutral pH, both $SO_4^{-\bullet}$ and $OH^{-\bullet}$ radicals contribute equally to pollutant degradation [396].

Literature on the degradation of TrOCs by activated PS is scarce, and to date has been generally focused on PS activation routes in the presence of a single TrOC. Previously, the combined peroxymonosulfate (50 µM) – Fe^{2+} (50 µM) process achieved above 99% degradation of atrazine, outperforming atrazine removal by coagulation [397]. Heat activated PS has been also reported to achieve 40-100% removal of a few investigated TrOCs such as atrazine, aniline, monochlorobenzene and 2,4-dichlorophenol [392]. Deng et al. [398] reported only 12% degradation of carbamazepine following 2 h treatment with heat-activated PS at a PS concentration and operating temperature of 1 mM and 40ºC, respectively. In a study by Ji et al. [399], PS (1 mM) activated by heat at 40ºC achieved 20% atrazine degradation after an incubation time of 120 h. Ji et al. [400] observed complete degradation of the antibiotic sulfamethoxazole within 8 h at 50ºC. These previous experiments were done in batch mode.

Instead of a single TrOC, performance of activated PS for the degradation of a wide range of TrOCs in their mixture should be assessed. In addition to TrOC degradation, PS activated by UV light was reported to control fouling during the treatment of surface water by an ultrafiltration membrane [401]. In another study by Chen et al. [397], fouling of an ultrafiltration membrane caused by humic substances and sodium alginate was significantly reduced by peroxymonosulfate activated by Fe^{2+}.

2.6. References

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Chapter 3: TrOC removal by a high retention nanofiltration vs. ultrafiltration enzymatic membrane bioreactor (UF- vs. NF-EMBR)

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Asif, M.B., Hou, J., Price, W.E., Chen, V., Hai, F.I. 2019. Nanofiltration vs ultrafiltration-coupled enzymatic membrane bioreactor (EMBR): Can the choice of a membrane influence trace organic contaminant degradation? Submitted to *Jouranl of Membrane Science*.

3.1. Introduction

Trace organic contaminants (TrOCs) such as pharmaceuticals, pesticides, steroid hormones and industrial chemicals are commonly detected in different environmental systems including surface water and groundwater due to the discharge of secondary treated wastewater [1, 2]. In addition, agricultural run-off, combined sewer overflow and stormwater run-off can significantly increase the concentration of TrOCs in freshwater bodies [2, 3]. Since TrOCs can be potentially harmful to the aquatic ecosystem and human health [4], an efficient treatment system is required for effective TrOC removal.

Conventional activated sludge process and membrane bioreactors (using micro- or ultrafiltration membranes) have been reported to be ineffective for the removal of TrOCs [5, 6]. Bioreactors equipped with high retention membranes (*e.g.,* nanofiltration or membrane distillation) can be a promising alternative. Among different types of high retention membrane separation processes, nanofiltration (NF) membranes have been studied extensively for the removal of TrOCs from secondary treated wastewater and freshwater [7-10]. Following membrane separation, an additional process is required for the treatment of the membrane-concentrate containing high concentrations of TrOCs. Instead of providing a separate treatment process for the degradation of TrOCs, it is a sensible approach to integrate a TrOC degradation process with the NF membrane. In this context, an enzymatic bioreactor can be combined with an NF membrane, which will provide complete retention and TrOC biodegradation in a single step. TrOC degradation by fungal enzymes in enzymatic bioreactors is a promising eco-friendly technique. Enzyme-catalyzed degradation of TrOCs by fungal enzymes does not produce secondary toxic sludge, which is a key attribute of physicochemical treatment processes [11, 12]. Among different fungal enzymes (*e.g.,* lignin peroxidases and manganese peroxidase), laccase is interesting as it does not require an external co-factor such as hydrogen peroxide (H_2O_2) to catalyze the degradation or oxidation of TrOCs [13]. Laccase-catalyzed degradation process typically involves the transfer of an electron from a substrate to the active sites of laccase followed by conversion of dissolved oxygen to water [14, 15]. The characteristics of active sites of laccase have been studied by using a combination of spectroscopic and crystallography techniques [16, 17]. Briefly, laccase active sites consist of four copper atoms, and can be classified into following categories: (i) Type 1 containing one copper atom; (ii) Type II containing one copper atom; and (iii) Type III containing a pair of copper atoms. During the degradation process, reduction of Type I copper site occurs due to the transfer of an electron from a substrate to the laccase. This promotes the transfer of an electron to Type II and Type III active sites where dissolved oxygen is reduced, and release of water takes place [15, 17].

Performance of laccase is governed by the operating conditions (*e.g.,* pH and temperature) and molecular properties of pollutants (*e.g.,* molecule structure and hydrophobicity). Typically, laccase can efficiently catalyze the degradation of TrOCs containing strong electron donating

functional groups (EDGs) such as hydroxyl (–OH) and amine (–NH) functional groups. By contrast, degradation of TrOCs containing strong electron withdrawing functional groups (EWGs) such as amide ($–NH_2$) and halogen (–X) is incomplete [11, 18, 19]. To improve the degradation of resistant TrOCs, a low molecular weight redox-mediator can be introduced in the enzymatic bioreactor. Redox-mediators are readily oxidized by laccase and produce highly reactive radicals that can either directly degrade or polymerize resistant TrOCs [20].

Performance of laccase for TrOC removal has been predominantly studied by operating the enzymatic bioreactor in batch mode to avoid enzyme washout. This issue has been addressed by coupling an ultrafiltration (UF) membrane to an enzymatic bioreactor to effectively retain the enzyme [21, 22]. Notably, the UF membrane integrated with an enzymatic membrane bioreactor (EMBR) can effectively retain laccase but are not expected to retain TrOCs *via* size exclusion. During the filtration of bioreactor media, an enzyme gel-layer was reported to be formed on the membrane surface, which partially retained the resistant TrOCs. Due to the prolonged contact time between TrOCs and laccase, the retained TrOCs were demonstrated to be degraded by laccase. This improved the overall performance of UF-EMBR [23]. Based on this observation, it can be envisaged that the simultaneous retention of laccase and TrOCs could facilitate degradation. However, this has not been systematically investigated by integrating an enzymatic bioreactor with a high retention NF membrane, which will retain both laccase and TrOCs, or a conventional UF membrane, which will only retain laccase but not TrOCs.

To-date, the performance of the NF based enzymatic membrane bioreactor (NF-EMBR) has been reported only once in available literature [24]. In the first study by Escalona et al. [24], removal of an industrial chemical (bisphenol A) by NF-EMBR was studied over a short duration of only 5 h by operating the NF-EMBR in full batch or recirculation mode [24]. Because the available studies focused on only one compounds, it is imperative to investigate the degradation of a broad spectrum of pollutants at the environmentally relevant concentration for elucidating the role of simultaneous TrOC and laccase retention on the performance of an EMBR.

In this chapter, the degradation of a set of 29 chemically diverse TrOCs in an enzymatic bioreactor coupled to the NF membrane (NF-EMBR) was assessed. To demonstrate the impact of effective TrOC retention on degradation, the performance of a "control" UF based EMBR that can only retain laccase but not TrOCs, was investigated and compared to that achieved by NF-EMBR. Importantly, the factors governing the performance of NF and UF membranes as well as laccase were studied. This facilitated in elucidating the mechanism responsible for better TrOC degradation in the NF-EMBR. To further improve the degradation of TrOCs, impact of a naturally occurring redox-mediator (violuric acid) at different concentrations was systematically studied. Finally, variations in membrane flux and changes in membrane properties were assessed and explained.

3.2. Hypothesis

- Simultaneous retention of laccase and TrOCs by the high retention NF membrane will facilitate degradation
- Physciochemical properties, particularly chemical structure may govern TrOC removal by laccase and membranes

3.3. Materials and methods

3.3.1. Enzyme solution, redox-mediator and trace organic contaminants

Laccase from genetically modified *Aspergillus oryzae* supplied by Novozymes Australia Pty. Ltd. (Sydney, NSW, Australia) was used. According to the supplier, w/w composition of enzyme solution was as follows: 66% water, 25% propylene glycol, 4% glucose, 3% laccase and 2% glycine. The purpose of adding propylene glycol, glucose and glycine is to stabilize the enzyme solution. The enzyme solution had an enzymatic activity of 190,000 $\mu M_{(DMP)}$/min, which was measured before the commencement of this experiment by using 2,6-dimethoxy phenol (DMP) as substrate at room temperature and pH =4.5 (*see* **Section 3.3.4.2**).

A naturally occurring redox-mediator, namely violuric acid (VA), was used because it has been reported to significantly improve degradation of TrOCs that are resistant to laccase-catalyzed degradation [25, 26]. Analytical grade VA was purchased from Sigma Aldrich (Sydney, NSW, Australia). A stock solution of VA was prepared in Milli-Q water and stored at -4 ºC in dark.

Various categories of TrOCs such as pharmaceuticals, personal care products, pesticides, steroid hormones and industrial chemicals are ubiquitously detected in freshwater bodies [2]. Therefore, synthetic wastewater was prepared by adding a mixture of 29 TrOCs in Milli-Q water at a concentration of 5 µg/L to stimulate the composition of TrOCs in environmental systems. These TrOCs include ten pharmaceuticals, seven pesticides, five naturally-occurring steroid hormones, three industrial chemicals, three ingredients of personal care products and one phytoestrogen (*see* **Appendix Table 3-1**). Relevant physicochemical properties of TrOCs are given in **Table 3.1**. Analytical grade TrOCs (purity >98%) were purchased from Sigma Aldrich (Sydney, NSW, Australia), and a stock solution containing the mixture of 29 TrOCs was prepared in methanol. The stock solution was stored at – 18 ºC in dark for use within one month.

Table 3.1. Physicochemical properties of the selected 29 TrOCs

TrOCs	Chemical formula	Molecular weight (g/mol)	Log D at pH=7	pK_a	Charge at pH=7
Non-phenolic TrOCs					
Clofibric acid	$C_{10}H_{11}ClO_3$	214.65	-1.06	3.18	-ve
Metronidazole	$C_6H_9N_3O_3$	171.15	-0.14	14.44	Neutral
Fenoprop	$C_9H_7Cl_3O_3$	269.51	-0.13	2.93	-ve
Ketoprofen	$C_{16}H_{14}O_3$	254.28	0.19	4.23	-ve
Naproxen	$C_{14}H_{14}O_3$	230.26	0.73	4.84	-ve
Primidone	$C_{12}H_{14}N_2O$	218.25	0.83	12.26	Neutral
Ibuprofen	$C_{13}H_{18}O_2$	206.28	0.94	4.14	-ve
Propoxur	$C_{11}H_{15}NO_3$	209.24	1.54	12.28	Neutral
Diclofenac	$C_{14}H_{11}Cl_2NO_2$	296.15	1.77	4.18	-ve
Carbamazepine	$C_{15}H_{12}N_2O$	236.27	1.89	13.94	Neutral
Gemfibrozil	$C_{15}H_{22}O_3$	250.33	2.07	4.75	-ve
Amitriptyline	$C_{20}H_{23}N$	277.4	2.28	9.18	Neutral
N, N-Diethyl-meta-toluamide (DEET)	$C_{12}H_{17}NO$	191.3	2.42	1.37	-ve
Atrazine	$C_8H_{14}ClN_5$	215.68	2.64	2.27	-ve
Ametryn	$C_9H_{17}N_5S$	227.33	2.97	3.71	-ve
Benzophenone	$C_{13}H_{10}O$	182.22	3.21	7.5	Neutral
Octocrylene	$C_{24}H_{27}N$	361.48	6.89	-	-
Phenolic TrOCs					
Salicylic acid	$C_7H_6O_3$	138.12	-1.13	3.01	-ve
Estriol	$C_{18}H_{24}O_3$	298.33	1.89	10.25	Neutral
Enterolactone	$C_{18}H_{18}O_4$	288.38	2.53	9.93	Neutral
Pentachlorophenol	C_6HCl_5O	266.34	2.85	4.68	Neutral
4-tert-Butylphenol	$C_{10}H_{14}O$	150.22	3.4	10.13	Neutral
Estrone	$C_{18}H_{22}O_2$	270.37	3.62	10.25	Neutral
Bisphenol A	$C_{15}H_{16}O_2$	228.29	3.64	10.29	Neutral
17α– Ethinylestradiol	$C_{20}H_{24}O_2$	269.4	4.11	10.24	Neutral
17β–Estradiol	$C_{18}H_{24}O_2$	272.38	4.15	10.27	Neutral
17β-Estradiol-17-acetate	$C_{20}H_{26}O_3$	314.42	5.11	10.26	Neutral
4-tert-Octylphenol	$C_{14}H_{22}O$	206.32	5.18	10.15	Neutral
Triclosan	$C_{12}H_7Cl_3O_2$	289.54	5.28	7.8	Neutral

Note: Data collected from SciFinder database, Taheran et al. [9]; and Fujioka et al. [27]

3.3.2. Experimental setup

A laboratory-scale cross-flow filtration system coupled to an enzymatic bioreactor (3 L) was used in this experiment (**Figure 3.1**). A detailed description of the filtration system is given elsewhere [27]. Briefly, this system mainly consists of a stainless-steel enzymatic bioreactor, high-pressure pump (Hydra-Cell, Wanner Engineering Inc., Minneapolis, MN, USA), stainless steel membrane cell, and bypass and back-pressure valves (Swagelok, Solon, OH, USA). The membrane cell with a channel height of 2 mm holds the flat-sheet NF or UF membrane with a

surface area of 40 cm^2. A digital flow meter (FlowCal, GJC Instruments Ltd, Chester, CH, UK) was connected to the permeate line for monitoring the permeate flux. The cross-flow velocity and temperature were maintained at 40.2 cm/s and 25 °C, respectively in all experiments.

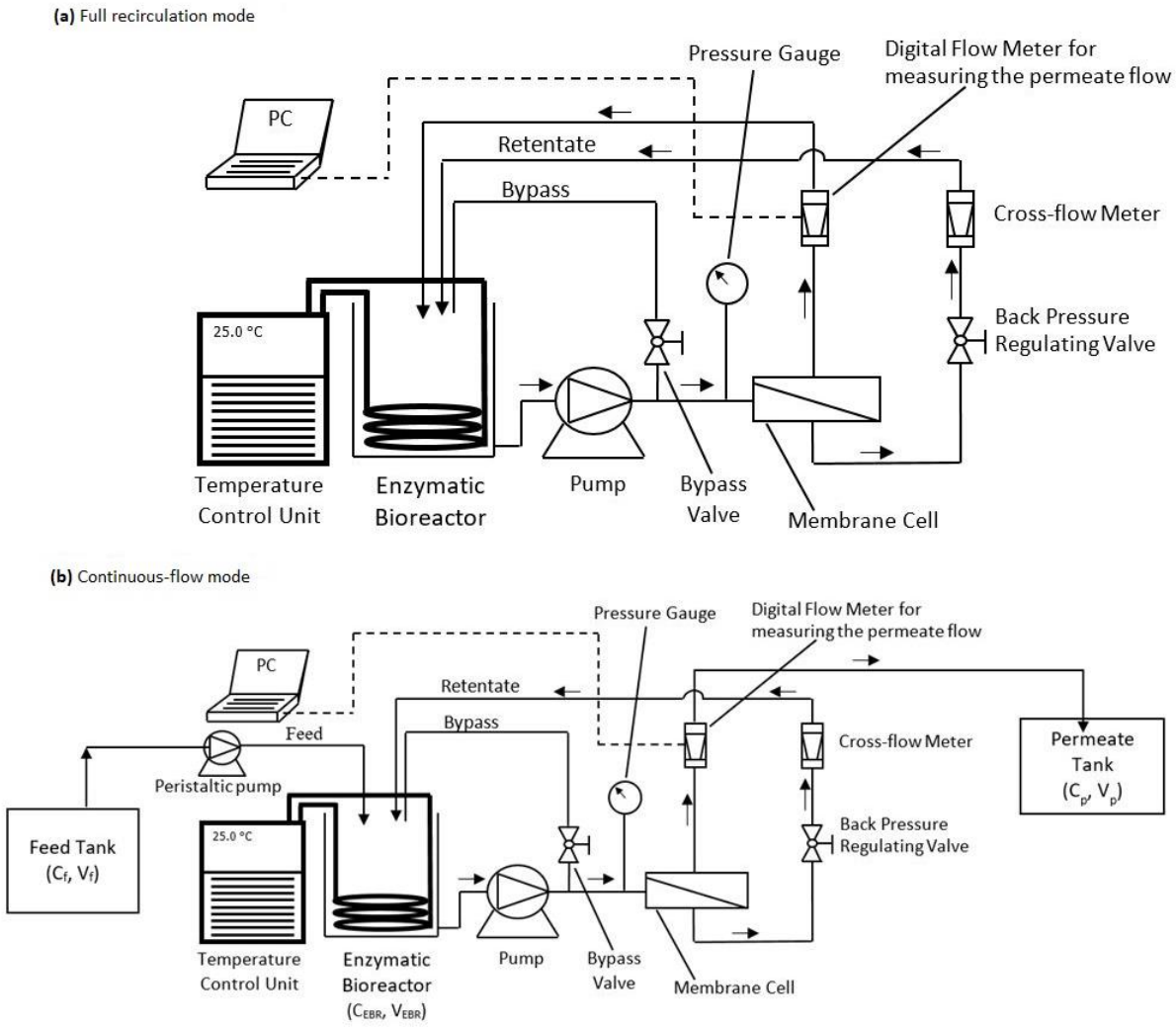

Figure 3.1. Schematics of the lab-scale cross-flow filtration system attached to an enzymatic bioreactor operated in full recirculation mode (a) and continuous-flow mode (b). Arrows show the direction of flow. Laccase retention was first confirmed with a short term (i.e., 24 h) study in full recirculation mode. Further operation of EMBRs were conducted in continuous-flow mode for assessing the impact of TrOC retention on their degradation. C_f, C_{EBR} and C_p are the concentration (µg/L) of a specific TrOC in the feed, enzymatic bioreactor and permeate, respectively. V_f, V_{EBR} and V_p represent the volume of feed, enzymatic bioreactor and permeate, respectively. A picture of lab-scale EMBR is shown in **Appendix Figure 3-2.**

Commercially available flat-sheet UF and NF membranes were used in this experiment. The UF membrane was purchased from Sterlitech (WA, USA). The active layer of the UF membrane is made of polyvinylidene fluoride (PVDF), and its molecular weight cut-off (MWCO) is 30,000 Da. The UF membrane was not expected to retain TrOCs by size exclusion, because the molecular weight of the selected TrOCs ranged between 138-361 Da (**Table 3.1**). On the other hand, the NF membrane (NF90, Dow chemicals, MI, USA) had an MWCO of 200

Da. It was a polyamide thin film composite (TFC) membrane that has been studied extensively for the rejection of recalcitrant pollutants from surface water and secondary treated wastewater [9]. However, the performance of the polyamide-TFC NF membrane has not been studied for the removal of TrOCs following its integration with an enzymatic bioreactor.

3.3.3. Enzymatic membrane bioreactor operation and experimental protocols

Each experiment was initiated with membrane compaction. The NF membrane was compacted at a pressure and cross-flow velocity of 10 bar and 40.2 cm/s, respectively, using Milli-Q water until the permeate flux stabilized at around 7 L/m^2 h. Similarly, the UF membrane was also compacted but without applying any pressure. This is because the cross-flow velocity of 40.2 cm/s was enough to generate a permeate flux equivalent to that achieved by the NF membrane. A series of experiments were conducted by operating UF/NF-EMBR separately to assess: TrOC degradation by laccase; and TrOC removal by the UF and NF membrane. This exeriment has two parts – (i) proof of the concept preliminary run: the performance of NF vs. UF coupled EMBRs for the degradation of only five TrOCs; and (ii) comprehensive run: the performance of NF vs. UF coupled EMBRs for the degradation of a broad spectrum of TrOCs. Experimental protocoals for both runs are explained separately in the following sections.

3.3.3.1. Preliminary assessment of NF vs. UF coupled EMBRs

The working volume of the enzymatic bioreactor was kept at 3 L in all experiments. In full recirculation mode, UF/NF-EMBRs were operated for a period of 24 h, and the membrane permeate was continuously returned back to the enzymatic bioreactor. The NF-EMBR was operated at a pressure of 8 bar and cross-flow velocity of 40.2 cm/s, which corresponds to an initial permeate flux of 6.9 L/m^2 h bar. Laccase was directly added to the enzymatic bioreactor to achieve an initial laccase activity of 180-185 $\mu M_{(DMP)}$/min. This laccase activity was selected based on that reported for previously developed UF-EMBRs [22, 28]. Stock solution containing the TrOC mixture was added to the enzymatic bioreactor to obtain a concentration of 1000 µg/L of each TrOC. However, the actual initial measured concentrations of atrazine, carbamazepine, sulfamethoxazole, diclofenac and oxybenzone were 1100±20, 1050±40, 1120±80, 1070±40 and 1000±30 µg/L (n=4), respectively.

All operating parameters for UF-EMBR were identical to that of NF-EMBR except the applied pressure as explained above. The EMBRs were first operated to confirm retention of laccase and TrOCs by the membrane and check the stability of laccase during EMBR operation. Duplicate samples were collected from the membrane permeate at 2, 4, 8 and 24 h for measuring laccase activity and TrOC removal.

All the operating conditions in continuous-flow mode were same as described above for full recirculation mode, except that the synthetic wastewater containing the mixture of TrOCs was continuously fed into the enzymatic bioreactors at a loading rate of 1.44 mg/L.d for each TrOC. A peristaltic pump (Masterflex, Vernon Hills, IL, USA) was used for continuous feeding.

Based on the initial permeate flux of the membranes (*i.e.,* 6.9 L/m^2 h bar), the hydraulic retention time (HRT) of the EMBRs was 16 h. The EMBRs were each operated continuously for a period of 48 h (*i.e.,* 3×HRT). During each run, duplicate samples were collected from the enzymatic bioreactor and membrane permeate at specific intervals (*i.e.,* 6, 12, 16, 24, 32, 38 and 48 h) for measuring laccase activity and TrOC removal. At the end of UF/NF-EMBR operation, the clean water flux was measured for 1 h using Milli-Q water to assess membrane fouling and flux recovery.

3.3.3.2. Assessment of NF vs. UF coupled EMBRs for broad spectrum of TrOCs

The NF-EMBR and UF-EMBR ("control") were operated under continuous mode to systematically investigate the effect of TrOC retention on their degradation. Under continuous mode, the synthetic wastewater containing the mixture of TrOC in ultrapure Milli-Q water was continuously fed to UF/NF-EMBR separately for a period of 68 h using peristaltic pumps (Masterflex, Vernon Hills, IL, USA). Based on the initial permeate flux of the membranes, the HRT for both EMBRs was approximately 16 h. Duplicate samples from enzymatic bioreactor were collected at 32 and 68 h for analysis to assess TrOC degradation by laccase. At same intervals (*i.e.*, 32 and 68 h), duplicate samples from permeate were also collected to analyze the overall removal of TrOCs (*i.e.*, biodegradation+membrane retention). At the end of each experiment, UF and NF membranes were backwashed with ultrapure Milli-Q water for 1 h, and the clean water flux of the membranes was measured to assess flux recovery.

Redox-mediators are low molecular weight phenolic compounds that can facilitate the degradation of TrOC by acting as an electron shuttle between laccase and target pollutant [20]. In this experiment, the NF-EMBR was operated with and without mediator dosing to investigate the influence of mediator dosing on TrOC degradation. A single dose of violuric acid (VA) was introduced at different concentrations (*i.e.,* 10, 25, 50 and 100 µM) separately to the NF-EMBR. Duplicate samples from enzymatic bioreactor and permeate were collected at 32 and 68 h for TrOC analysis.

3.3.3.2.4. Laccase stability and maintenance in EMBRs

During the operation of EMBRs, laccase activity may diminish due to various physical, chemical and biological inhibitors such as shear stress caused by membrane filtration [13, 29]. Moreover, the transformation products formed following TrOC degradation in an EMBR can also inhibit laccase by blocking the active sites of enzymes [30]. Therefore, laccase activity was regularly monitored during the operation of EMBRs. Based on laccase activity drop (*see* **Appendix Figure 3-3**), a protocol of re-injecting a small dose of laccase (250 µL per litre of bioreactor media) was developed to maintain a laccase activity of 170-185 $\mu M_{(DMP)}$/min for stable TrOC degradation.

3.3.4. Analytical methods

3.3.4.1. TrOC analysis

During prelimnary assessment with five compounds, quantification of TrOCs was carried out by using High-performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan) at the detection wavelength of 280 nm using a method reported previously [31]. Briefly, the HPLC system was equipped with a UV-Vis detector and C-18 column (300×4.6 mm) having a pore size of 5 µm (Supelco Drug Discovery, Sigma Aldrich, Sydney, NSW, Australia). Milli-Q water buffered with 25 mM KH_2PO_4 and HPLC grade acetonitrile were used as the mobile phase for TrOC quantification. Two eluents, namely eluent A (20% acetonitrile + 80% buffer, v/v) and eluent B (80% acetonitrile + 20% buffer, v/v), were passed through the C-18 column at a flow rate of 0.7 mL/min for 30 min in time dependent gradients as follows: [Time (min), A (%)]: [0, 85], [8, 40], [10, 0], [22, 0], [24, 85]. The gradient of eluent B was then automatically adjusted as follows: [Time (min), B (%)]: [0, 15], [8, 60], [10, 100], [22, 100], [24, 15]. The limit of detection (LOD) for this method was approximately 10 µg/L.

During experiment with as broad spectrum of 29 compounds, TrOC concentration was measured using a method previously described by Hai et al. [32]. This method involves the extraction of TrOC by solid-phase extraction technique followed by their quantification using a GC/MS system (QP5000, Shimadzu, Japan). TrOCs present in the feed, supernatant and permeate samples were extracted using 6 mL 200 mg Oasis HLB cartridges (Waters, Milford, MA, USA). The TrOC extraction procedure was as follows: (i) pre-conditioning of HLB cartridge with 5 mL dichloromethane and methanol solution (1:1 v/v), 5 mL methanol and 5 mL Milli-Q water; (ii) loading of acidified (pH 2-2.5) samples onto the cartridges at a flow rate of 1–4 mL/min; and (iii) drying of cartridges with nitrogen for 30 min. The TrOCs were subsequently eluted from the cartridge using 5 mL of methanol followed by dichloromethane and methanol mixture (1:1 v/v) at a flow rate of 1–4 mL/min. The effluent was subsequently evaporated at 40 ^{o}C under a gentle stream of nitrogen. The residual after evaporation was re-dissolved in 200 µL methanol containing an internal standard (5 mg bisphenol A-d16) before its transfer into 1.5 mL vials. The mixture present in 1.5 mL vials was again evaporated under gentle stream of nitrogen. Finally, the extracts were derivatized by adding 100 µL of N,O-bis(trimethylsilyl)trifluoroacetamide (1% trimethylchlorosilane) and pyridine (dried with KOH solid), then heated on a heating block (60–70 ^{o}C) for 30 min. The derivatives were cooled to room temperature and analyzed using the Shimadzu QP5000 GC–MS (Shimadzu, Kyoto, Japan). The limit of detection (LOD) for this method is compound specific and ranged from 1-20 ng/L as listed in **Appendix Table 3-1**. Removal efficiency by laccase ($R_{degradation}$) and the membrane ($R_{membrane}$) was measured as:

$$R_{degradation} = 100 \times (1 - C_{EBR}/C_f) \quad (1)$$

$$R_{membrane+degradation} = 100 \times (1 - C_p/C_f) \quad (2)$$

where, C_f, C_{EBR} and C_p are the concentration (ng/L) of a specific TrOC in feed, enzymatic bioreactor and permeate, respectively. The mass of TrOCs degraded by laccase was calculated as follows:

$$C_f \times V_f = (C_{EBR} \times V_{EBR}) + (C_p \times V_p) + \text{biodegradation/biotransformation} \quad (3)$$

where, V_f, V_{EBR} and V_p represents the volume of feed, enzymatic bioreactor and permeate, respectively.

3.3.4.2. Laccase activity assay and ORP

Laccase activity was measured by using a method previously reported by Paszczynski et al. [33]. Briefly, the change in absorbance of 2,6-dimethoxyl phenol (DMP) in sodium citrate buffer (pH = 4.5) was recorded over a duration of 2 min at room temperature using a UV-Vis spectrometer (DR3900, HACH, Loveland, Colorado, USA). A molar extinction coefficient of 49.6/mM cm was used to calculate laccase activity. Oxidation-reduction potential (ORP) of laccase with and without the addition of redox-mediator was measured using an ORP meter (WP-80D dual pH-mV meter, Thermo Fisher Scientific, Australia).

3.3.4.3. Analysis of membrane properties and surface morphology

Surface charge and hydrophobicity was analyzed to assess the effect of laccase on membrane properties. Membrane hydrophobicity in terms of contact angle was measured by the standard sessile drop method using a Rame-Hart Goniometer (Model 250, Rame-Hart, Netcong, New Jersey, USA) as previously described [34].

For assessing the change in surface charge of the membranes, the zeta potential was measured at room temperature using a SurPASS elctrokinetic analyzer (Anton Par GmbH, Graz, Austria). Analytical grade potassium hydroxide and hydrochloric acid were used to adjust the pH of the electrolyte solution. The zeta potential was calculated from the steaming potential using the Fairbrother-Mastin approach [34].

NF and UF membranes collected at the end of experiments were air-dried in a desiccator. After coating the membranes with a gold layer by using a sputter coater (SPI Module, West Chester, PA, USA), the surface morphology of the membranes was characterized with scanning electron microscopy (SEM) (JCM-600, JEOL, Tokyo, Japan).

3.4. Results and discussion

3.4.1. Laccase and TrOC retention by the membranes

Coupling a membrane to the enzymatic bioreactor can prevent washout of the enzyme along with treated effluent. The flat-sheet UF and NF membranes used in this experiment have not been tested before for laccase retention. Hence, effective retention of laccase was studied by operating UF/NF-EMBRs in full recirculation mode. Laccase activity in NF-EMBR permeate remained undetected throughout operation as shown in **Figure 3.2**, thus confirming effective

retention of laccase by the NF membrane. On the other hand, no laccase activity was detected in the permeate during the first 4 h of UF-EMBR operation in full recirculation mode, but a small laccase activity of 5-7 $\mu M_{(DMP)}$/min (*i.e.*, still above 95% laccase retention) was measured in UF-permeate samples for the rest of the experiment. In previously developed UF-EMBR, hollow fiber UF membranes with molecular weight cutoff (MWCO) of 6-10 kDa effectively retained laccase in the enzymatic bioreactor [21, 22]. Although the MWCO of the flat-sheet UF membrane (30 kDa) used in this experiment was smaller than the size of laccase (56 kDa), slight passage of laccase through the UF membrane can be attributed to its diffusion into the permeate following the formation of a laccase gel-layer on the active side of membrane that was visible to the naked eye. The enzyme gel-layer formed on the UF membrane can be seen in the picture given in **Appendix Figure 3-4**. In addition, membrane pore size may be non-uniform, and presence of pores with diameter greater than the average pore size can increase the effective MWCO of a membrane. Furthermore, depending on water matrix (*e.g.*, ionic strength and pH) and membrane properties (*e.g.*, surface charge, hydrophobicity and pore size), chemicals may permeate even through the membrane with a smaller MWCO. Similar observations were made when two enzymes, namely, lysozyme and protease were concentrated using polysulfone and polyethersulfone ultrafiltration membranes, respectively [35, 36].

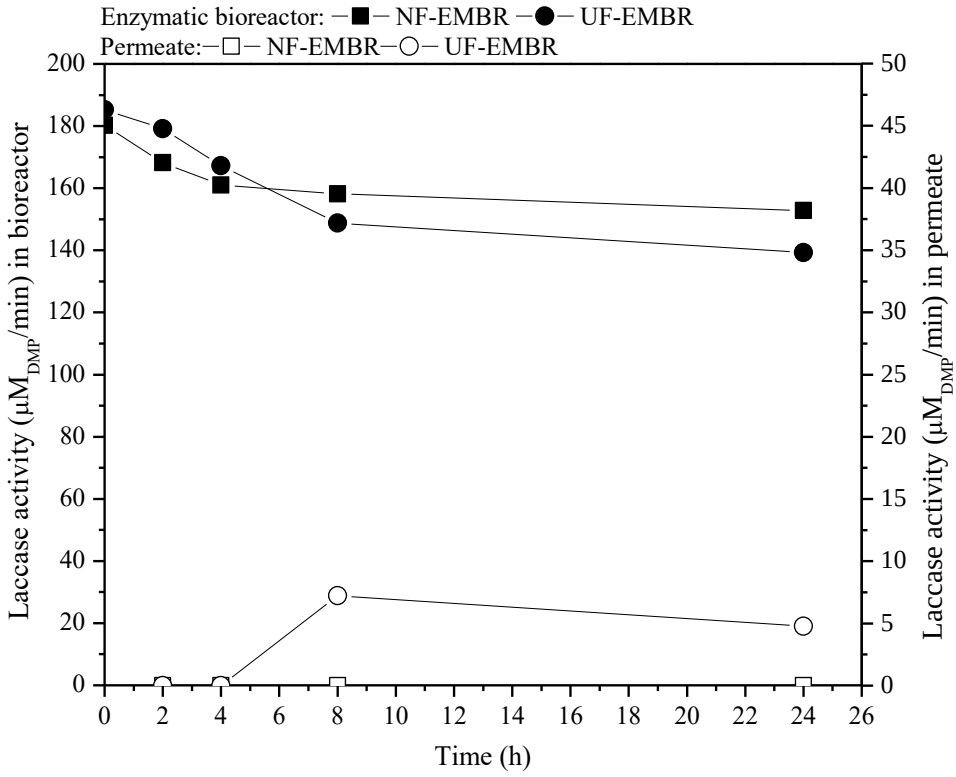

Figure 3.2. Laccase activity in the enzymatic bioreactor and permeate of UF-EMBR and NF-EMBR during their operation in full recirculation mode for 24 h. The standard deviation of duplicate samples was less than 2%.

The results of NF-EMBR operation in full recirculation mode confirmed above 95% retention of the TrOCs by the NF membrane. Conversely, TrOC rejection by the UF membrane varied

between 1% (Sulfamethoxazole) and 5% (diclofenac). The rejections of TrOCs by both membranes are shown in **Appendix Figure 3-5**.

3.4.2. Preliminary assessment of TrOC removal in UF vs. NF-EMBRs

In this experiment, NF-EMBR achieved 92 to over 99% removal of the TrOCs (**Figure 3.3**). In general, NF membranes can remove TrOCs *via* the following mechanisms: (i) size exclusion; (ii) adsorption; and (iii) electrostatic interaction [9, 37]. TrOCs having a molecular weight higher than 200 g/mol have been reported to be effectively rejected by the NF90 membrane [38]. Because the molecular weight of the selected TrOCs during preliminary assessment was above 200 g/mole, effective rejection (92-99%) could be attributed to size exclusion mechanism. Moreover, charge repulsion between the negatively charged NF membrane (**Table 3.1**) and negatively charged TrOCs (*i.e.*, diclofenac, sulfamethoxazole and atrazine) could be responsible for their rejection by the NF membrane in the NF-EMBR. Adsorption of hydrophobic TrOCs (log D>3.2), which are generally neutral at pH=7, on membrane surface has been reported to result in effective rejection by the NF membrane at the initial stage of operation. However, their rejection could reduce gradually with time due to the diffusion of hydrophobic TrOCs into permeate [9, 39]. In this experiment, the NF-EMBR achieved above 99% removal of a hydrophobic TrOC, namely oxybenzone (log D = 3.99), because it was highly degraded (~99%) by laccase (**Figure 3.4**).

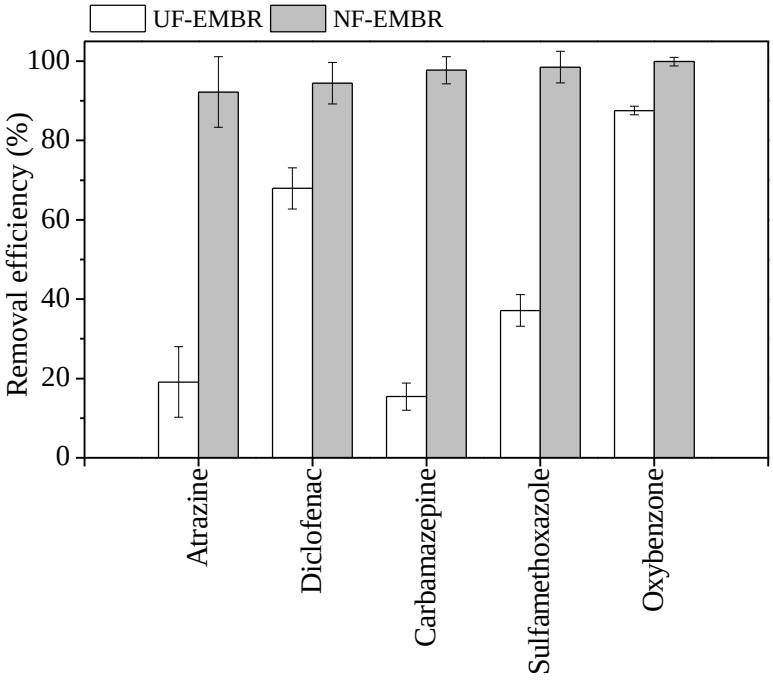

Figure 3.3. Overall removal (*i.e.*, degradation + membrane retention) of TrOCs in UF- and NF-EMBRs operated separately at an HRT of 16 h and TrOC loading rate of 1.44 mg/L d. Data presented as average ± standard deviation (n = 6).

The overall removal of the TrOCs by the NF-EMBR (as indicated by TrOC concentration in the membrane permeate) was 10-80% higher than that by the UF-EMBR (**Figure 3.3**). UF

membranes are not expected to remove TrOCs *via* size exclusion. However, it was observed that TrOCs were partially retained by the UF membrane. This aspect has been comprehensively discussed in **Section 3.4.3.3**.

During preliminary assessment of TrOC degradation by laccase in EMBRs, remaining TrOCs following degradation will mostly pass through the membrane (for UF membrane) or be significantly retained (for NF membrane). The NF membrane is expected to retain TrOCs more effectively than the UF membrane, but the current experiment seeks to assess if the application of NF can also enhance degradation. The UF/NF-EMBRs were continuously operated for a duration of 3×HRT under identical conditions to provide a common basis for comparing the degradation of TrOCs in UF- and NF-EMBRs. The degradation of TrOCs by laccase in UF/NF-EMBR was calculated using Equation (1). Among the selected TrOCs, efficient degradation (80-99%) of oxybenzone was achieved by laccase in both UF- and NF-EMBRs (**Figure 3.4**). In addition, its degradation was observed to be above 50% within the first 6 h of EMBR operation, which suggested that oxybenzone was easily amenable to degradation by laccase. Since phenols are typical substrates of laccase [11, 13], high removal of oxybenzone by laccase could be attributed to the presence of a phenolic moiety in its molecule. Indeed, oxybenzone removal by batch and continuous-flow enzymatic bioreactors has been reported to range from 60-99% [40, 41]. Gago-Ferrero et al. [42] reported the formation of three degradation products, namely benzophenone-1, 4-hydroxybenzophenone and 4,4′-dihydroxybenzophenone, following laccase-mediated degradation of oxybenzone. Interestingly, despite being inherently amenable to laccase-catalyzed degradation, its degradation was 19% better in NF-EMBR as compared to UF-EMBR (**Figure 3.4**). This could be attributed to the effective retention of oxybenzone by the NF membrane, which resulted in its prolonged interaction with laccase.

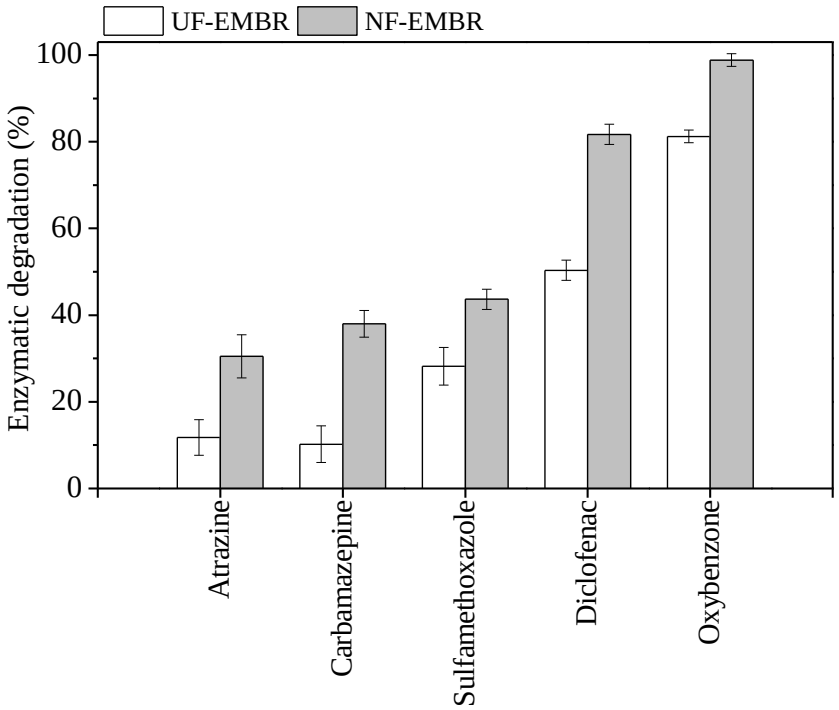

Figure 3.4. Degradation of TrOCs in UF- and NF-EMBRs operated separately at an HRT of 16 h and TrOC loading rate of 1.44 mg/L d. Data presented as average ± standard deviation (n = 6).

During preliminary assessment, compared to the UF-EMBR, better degradation (15-30%) of the non-phenolic TrOCs was achieved by the NF-EMBR (**Figure 3.4**). For example, degradation of atrazine and carbamazepine was 29 and 35%, respectively, by the NF-EMBR, while their degradation was approximately 10% in UF-EMBR. Similarly, degradation of sulfamethoxazole and diclofenac was 10-30% better as compared to that achieved by UF-EMBR (**Figure 3.4**).

Literature on the performance of an NF based enzymatic membrane bioreactor for TrOC removal is scarce. To date, only one study [24] has reported the performance of laccase in an NF-EMBR in the recirculation mode (rather than the continuous flow, which is required for scaling up) for a period of only 5 h and targeting only one TrOC (*i.e.*, bisphenol A). To improve from the previous study by Escalona [24], in this chapter, degradation of a mixture of TrOCs by laccase was assessed by operating the NF-EMBR in continuous-flow mode for a longer duration of 3 × HRT (*i.e.*, 48 h). Indeed, long term operation of a bioreactor is critical to achieve steady state TrOC degradation (**Figure 3.5**). The results of the preliminary assessment suggest that a NF membrane-coupled enzymatic bioreactor cannot only produce high quality effluent due to effective TrOC retention, but also achieve improved TrOC biodegradation (*i.e.*, reduced concentrate disposal). However, it is imperative to investigate the degradation of a broad spectrum of pollutants in NF-EMBR as demonstrated in next section.

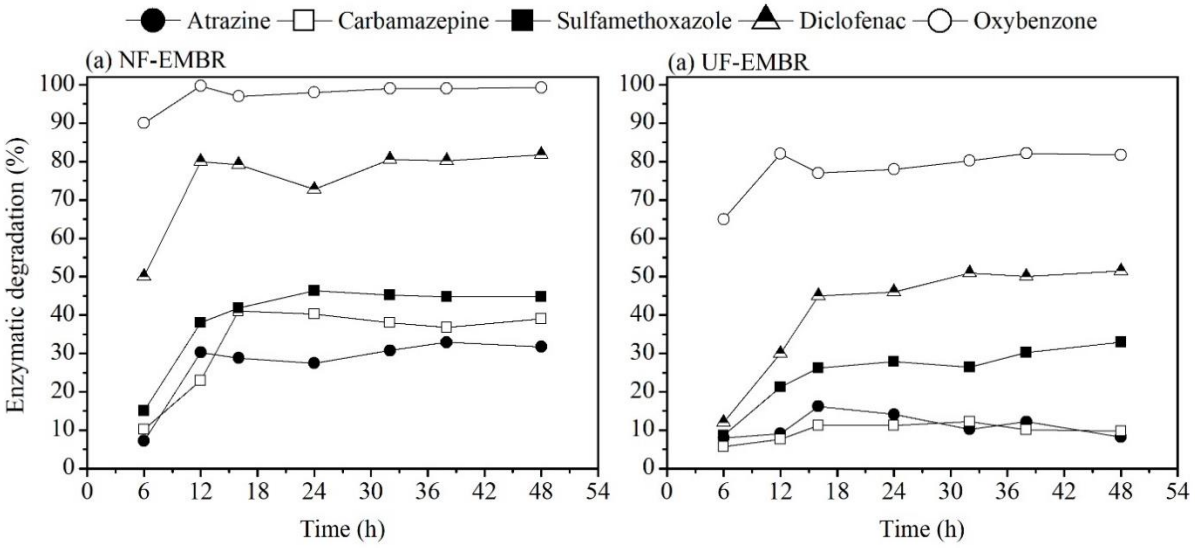

Figure 3.5. Time course of TrOC degradation by laccase in continuous-flow UF- and NF-EMBRs. Each data point denotes average of two samples with a variation of less than 5%.

3.4.3 Laccase-catalyzed degradation of a broad set of TrOCs by NF-EMBR

During assessment of EMBRs for the degradation of a broad spectrum of TrOC, laccase was again effectively retained (>95%) by both the membranes (**Appendix Figure 3-6**). TrOCs were retained effectively only by the NF membrane, and this was confirmed by filtering a TrOC mixture in Milli Q water through the membrane (**Appendix Figure 3-7**). Hence, the operating conditions of the UF/NF-EMBRs were suitable to systematically investigate the impacts of effective TrOC retention within the enzymatic bioreactor on their laccase-catalyzed degradation.

Laccase-catalyzed degradation occurs due to the transfer of a single electron from a substrate to laccase [20]. With some exceptions, phenolic TrOCs have been reported to be effectively degraded by laccase [13, 43]. On the other hand, degradation of non-phenolic TrOCs by laccase can be highly variable and may depend on the difference of ORP between laccase and the non-phenolic TrOCs as well as the TrOC molecular properties such as the presence of an EWGs or EDGs [11, 44]. Therefore, here the degradation of the phenolic and non-phenolic TrOCs is discussed separately.

3.4.3.1. Degradation of phenolic TrOCs

In this experiment, laccase achieved efficient degradation (>80%) for four out of 12 phenolic TrOCs, namely 17ß–estradiol-17-acetate, 4-tert-octylphenol, triclosan and salicylic acid in both UF- and NF-EMBRs (**Figure 3.6**). Efficient degradation of these TrOCs by laccase has been reported previously in both batch and continuous-flow enzymatic bioreactors [23, 45, 46].

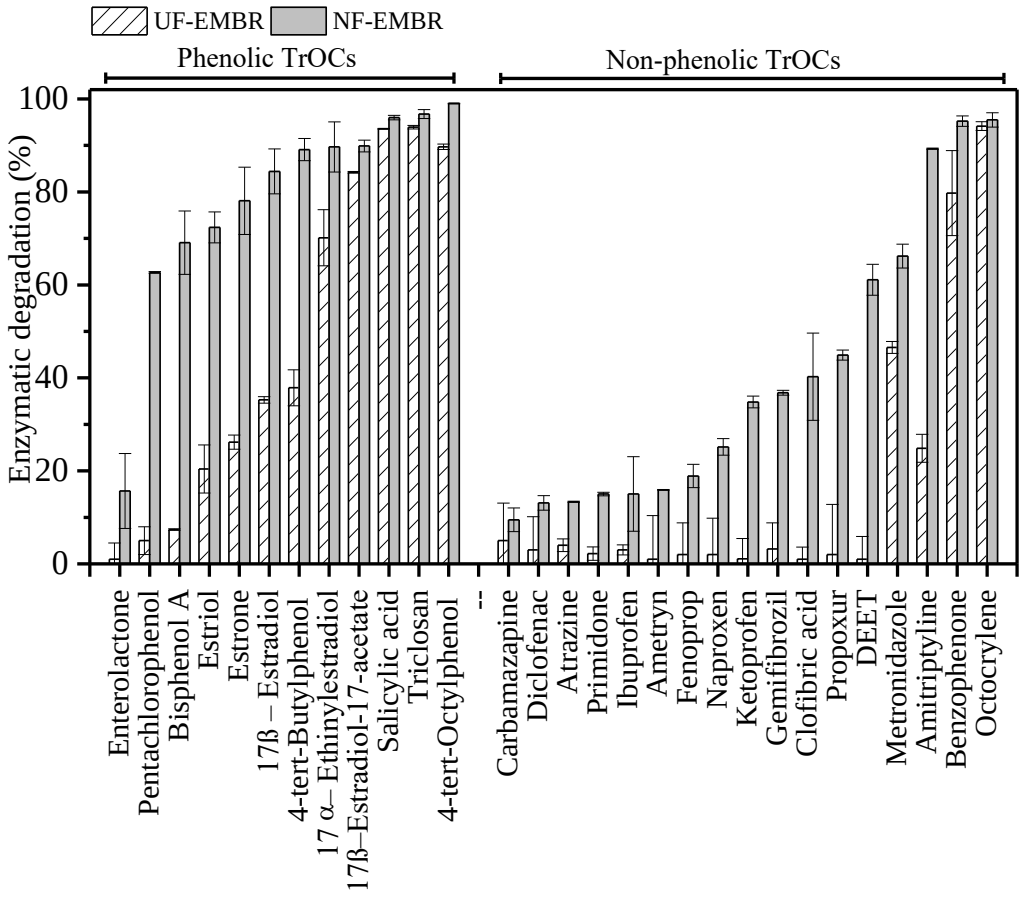

Figure 3.6. Degradation of TrOCs in enzymatic bioreactor coupled to the UF or NF membrane for showing the effect of effective TrOC retention on degradation. Both enzymatic membrane bioreactors were operated at an initial laccase activity of 180 $\mu M_{(DMP)}$/min, TrOC concentration of 5 µg/L, HRT of 16 h and cross-flow velocity of 40.2 cm/s. The temperature of the enzymatic bioreactor was kept at 25 ºC. Data is presented as average ± standard deviation (n=4).

As mentioned above, phenolic pollutants are typical substrates of laccase. However, the concomitant presence of EWGs in the molecule of phenolic TrOCs can cause steric hindrance, thereby delaying the access of a pollutant to the active sites of laccase for effective degradation [44]. For phenolic TrOCs containing EWG(s), the extent of degradation by laccase in NF-EMBR was observed to vary depending on the type of EWGs. For example, NF-EMBR achieved 80% degradation of estrone that contains the carbonyl (=O) functional group as an EWG in its molecular structure. On the other hand, degradation of pentachlorophenol, containing a halogen (–X) functional group as an EWG, was observed to be 60% in NF-EMBR.

Notwithstanding the above-mentioned variations in the degradation of the phenolic TrOCs containing EWG(s), NF-EMBR achieved from 5 up to 60% better degradation for eight out of the 12 investigated phenolic TrOCs as compared to the UF-EMBR (**Figure 3.6**). When an NF membrane is attached to an enzymatic bioreactor, the HRT of the bioreactor can be decoupled from the organic retention time due to effective TrOC retention. This leads to increased contact time between laccase and TrOC and can thus facilitate TrOC degradation. Indeed, in a study

by Lloret et al. [21], enhanced removal (33-37%) of two phenolic TrOCs, namely estrone and 17ß–estradiol, was achieved by increasing the HRT of an enzymatic bioreactor coupled to a UF membrane by 2 h. It is important to note that prolonged contact time might not be the only reason for improved degradation of TrOCs in the NF-EMBR: phenolic TrOCs (*e.g.*, bisphenol A), which could act as a redox-mediator, can also improve the degradation of TrOCs in NF-EMBR. This aspect is discussed further in **Section 3.4.3.2**.

Degradation of six phenolic TrOCs including four steroid hormones (17ß–estradiol and 17α–ethinylestradiol and estriol) and two industrial chemicals (4-tert-butylphenol and bisphenol A) by laccase was 70-90% in NF-EMBR, while UF-EMBR achieved 10-40% degradation (**Figure 3.6**). Although these TrOCs have been generally reported to be well removed by laccase in batch enzymatic bioreactors [46, 47], the lower performance of UF-EMBR in this experiment can be attributed to the continuous TrOC loading that has been reported to affect the extent of TrOC degradation [21, 23]. Continuous TrOC loading in UF-EMBR could affect the extent of TrOC degradation due to kinetic limitations. Under sustained TrOC loading, TrOCs occupy all the active sites of laccase, eventually reducing the percent degradation [23, 44]. Notably, the NF-EMBR was better suited to withstand the continuous loading of the phenolic TrOCs to the enzymatic bioreactor.

3.4.3.2. Degradation of non-phenolic TrOCs

In this experiment, the following three trends were observed in the degradation profile of 17 non-phenolic TrOCs (**Figure 3.6**): (i) From 5 up to 65% better degradation of six pharmaceuticals (*i.e.*, ketoprofen, naproxen, primidone, gemfibrozil, amitriptyline and metronidazole) and five pesticides, namely fenoprop, clofibric acid, propoxur, pentachlorophenol, N, N-diethyl-meta-toluamide (DEET) and ametryn, in NF-EMBR as compared to UF-EMBR; (ii) efficient degradation (>80%) of two ingredients of personal care products (*i.e.*, benzophenone and octocrylene) in both UF- and NF-EMBR; and (iii) poor removal (5-15%) of a pesticide (*i.e.*, atrazine) and three pharmaceuticals (*i.e.*, carbamazepine, diclofenac and ibuprofen) in both UF- and NF-EMBR.

Only around 5-15% degradation of atrazine, carbamazepine, diclofenac and ibuprofen by the EMBRs can be attributed to the presence of strong EWGs such as amide ($–NH_2$), carboxylic (–COOH) and halogen (–X) functional groups (*see* **Appendix Table 3-1**), which makes them resistant to laccase-catalyzed degradation [11, 19, 47]. On the other hand, benzophenone and octocrylene, which were well removed by laccase in this experiment, were also reported to be efficiently degraded by laccase in a continuous-flow enzymatic bioreactor [23].

The significantly better degradation of 11 non-phenolic TrOCs following their retention within the NF-EMBR can be attributed to the increased reaction time between laccase and the pollutants. Asif et al. [26] reported high TrOCs degradation in membrane distillation (MD)-EMBR, where the studied TrOCs and laccase were retained by MD membrane. However, in

that experiment the performance of the MD-EMBR was not compared to a suitable "control" *i.e.*, an EMBR that will retain laccase but not the TrOCs [26]. By comparing UF- vs. NF-EMBR, this experiment demonstrates that effective TrOC retention within the bioreactor facilitates their degradation.

It is important to note that TrOCs containing hydroxyl and amine functional groups such as bisphenol A and steroid hormones can also play an important role in the degradation of non-phenolic TrOCs by acting as redox-mediators [46, 48]. The secondary radicals or coupling agents, which are formed following the oxidation of TrOCs containing hydroxyl and amine functional groups, are highly reactive and could directly oxidize or polymerize other TrOCs. For instance, lignin is a plant polymer with a highly complex chemical structure. The degradation pathway for lignin reveals that laccase directly oxidizes the phenolic components of lignin, and produces highly reactive phenoxyl radicals, which then oxidize the non-phenolic components of lignin [44, 49]. Similarly, Hachi et al. [48] demonstrated that the degradation of acetaminophen by laccase formed a coupling agent (*i.e.*, dimer). This coupling agent reacted with carbamazepine to form oligomers, thereby improving carbamazepine removal from 10 to 40% [48]. In another study by Jahangiri et al. [50], removal of triclosan was reported to improve in a batch enzymatic bioreactor following the addition of the phenolic compound acetaminophen. Enhanced removal of triclosan was attributed to the formation of acetaminophen-triclosan cross-coupling products [50]. In the current experiment, the synthetic wastewater that was continuously fed to the UF/NF-EMBR contained a mixture of 29 TrOCs including 12 phenolic and 17 non-phenolic TrOCs. Since these TrOCs were effectively retained by the NF membrane but not by the UF membrane (*see* **Section 3.4.3.3**), it is possible that the radicals or coupling agents formed after the oxidation of some phenolic TrOCs by laccase contributed to better degradation of the non-phenolic TrOCs in NF-EMBR as compared to UF-EMBR. A close look at the trend of laccase-catalyzed degradation in both UF- and NF-EMBR indicates that the improvement in degradation could be correlated with the molecular weight of TrOCs. In the current experiment, the extent of improvement in degradation was significantly higher for TrOCs with a molecular weight above 200 g/mol (**Figure 3.7**). This is probably because the presence of more branches and/or functional groups in TrOCs with high molecular weight would create more opportunities of their interaction with laccase, secondary radicals and coupling agent [51].

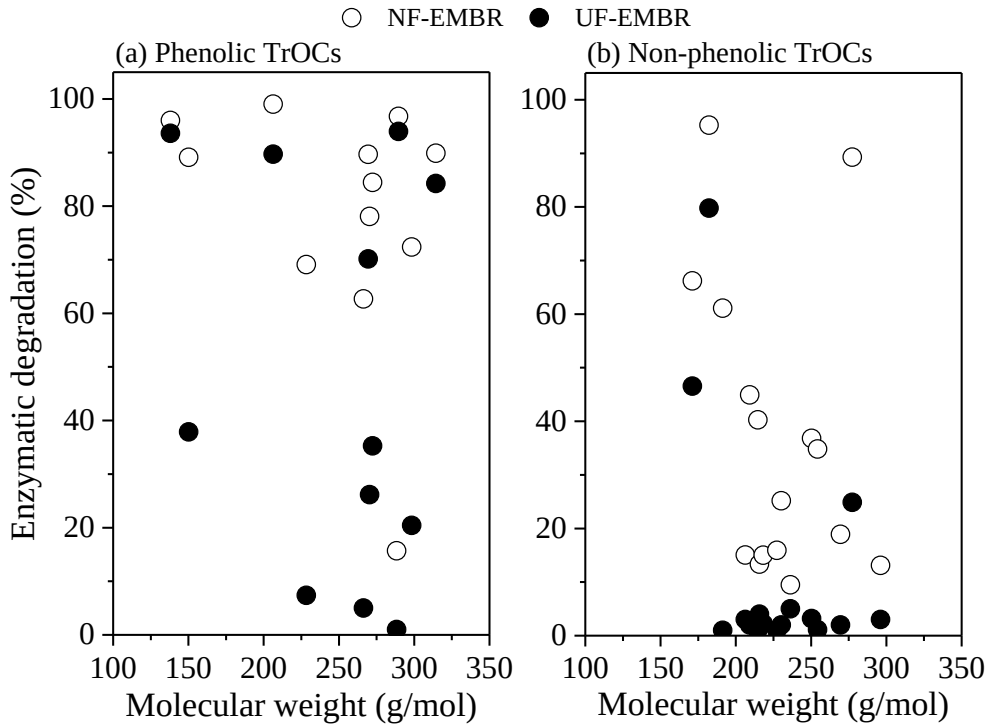

Figure 3.7. Enzymatic degradation in both UF- and NF-EMBR as function of TrOC molecular weight, showing that the extent of degradation was significantly higher for TrOCs with a molecular weight above 200 g/mol.

3.4.3.3. Overall removal of TrOCs in EMBRs

TrOC degradation in the enzymatic bioreactor ranged between 10-99% (**Figure 3.6**). However, the overall TrOC removal (calculated based on TrOC concentration in membrane permeate) by the NF-EMBR was 90-99%, demonstrating the significant contribution of the NF membrane to the overall removal.

As explained in **Section 3.4.2**, NF membranes can reject TrOCs *via* following mechanisms: (i) size exclusion; (ii) electrostatic interaction; and (iii) adsorption. In general, the NF membrane used in this experiment has been reported to effectively retain TrOCs with a molecular weight of greater than 200 g/mol (*i.e.*, MW > MWCO) *via* size exclusion mechanism [38]. With a few exceptions, the molecular weight of the TrOCs investigated was greater than 200 g/mol, and indeed they were effectively removed (>90%) by the NF-EMBR (**Figure 3.8**). The exceptions include salicylic acid (138.12 g/mol), metronidazole (171.15 g/mol), benzophenone (182.22 g/mol), DEET (191.27 g/mol) and 4-tert-butylphenol (150.22 g/mol). Removal of these TrOCs also ranged between 95-99% in NF-EMBR (**Figure 3.8**). Since salicylic acid, atrazine and DEET are negatively charged (pK_a < pH) at the operating pH of the NF-EMBR (*i.e.*, 6.7-6.9), charge repulsion between the negatively charged NF membrane and anionic TrOCs is likely responsible for their removal by the NF membrane [9, 27, 52].

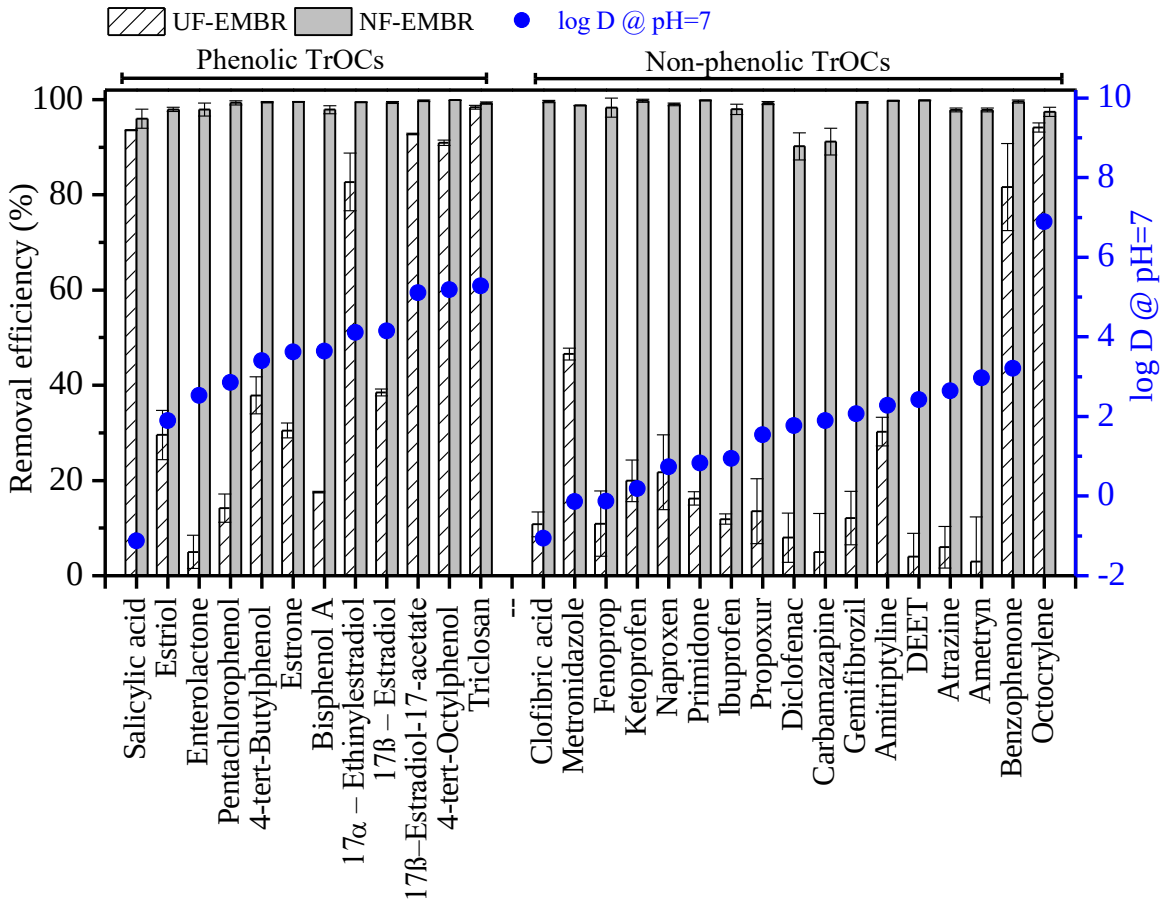

Figure 3.8. Overall TrOC removal in enzymatic bioreactor coupled to the UF or NF membrane. Data is presented as the average ± standard deviation (n=4).

TrOC adsorption on the membrane surface is also a mechanism of removal by NF membranes. Hydrophobic TrOCs (log D > 3) can adsorb on the membrane surface, thereby resulting in their high initial rejection by the NF membrane [9, 53], but this may reduce with time due to their diffusion into membrane permeate [9, 54]. Nevertheless, in this experiment, the hydrophobic TrOCs were efficiently degraded by laccase in the enzymatic bioreactor (80-99%, **Figure 3.6**). Thus, the overall removal of hydrophobic TrOCs (log D > 3) was above 99%. Previous studies reported that a combination of activated sludge [55] or enzymatic bioreactor [26] with a high retention membrane (*e.g.*, MD membrane) can improve the overall removal of TrOCs compared to a stand-alone high retention membrane system. However, this is the first study that demonstrates the performance of an NF-based EMBR for a set of 29 TrOCs.

UF membranes cannot reject TrOCs *via* size exclusion. Thus, as expected, the overall TrOC removal by the NF-EMBR was 10-80% higher than the UF-EMBR (**Figure 3.8**). However, it is noteworthy that, for the UF-EMBR, the overall removal efficiency of a few TrOCs was significantly better than that suggested by biodegradation efficiency (**Figure 3.6**). This indicates that the UF membrane provided partial retention of those TrOCs. To facilitate the discussion on TrOC removal by the UF membrane, the ratio of the concentration of selected

TrOCs in membrane permeate and bioreactor (*i.e.*, permeate/bioreactor ratio) is shown in **Figure 3.9**.

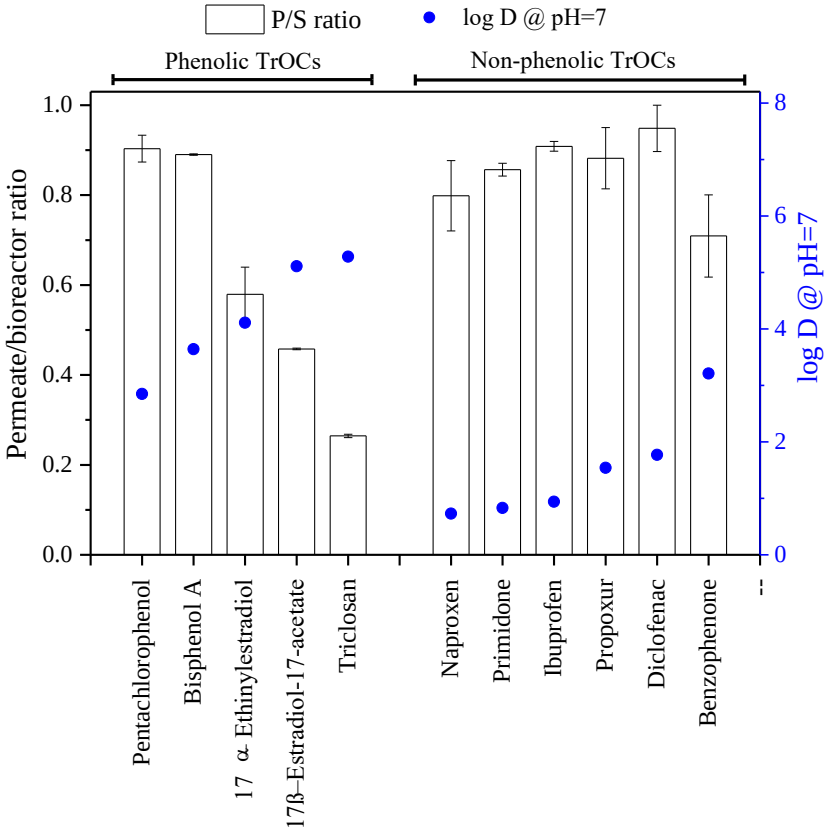

Figure 3.9. Permeate to supernatant (P/S) ratio of the selected TrOCs to show their partial retention by the UF membrane in UF-EMBR. Data is presented as average ± standard deviation (n=4).

Indeed, the permeate/bioreactor concentration ratio for significantly hydrophobic TrOCs (log D>3) including 17ß–estradiol-17-acetate, triclosan and 17α-ethinylestradiol was significantly below 1 and ranged between 0.3-0.6 (**Figure 3.9**). Previously, Nguyen et al. [23] observed adsorption of TrOCs on the enzyme gel-layer formed on the surface of a polyacrylonitrile hollow fiber UF membrane following the filtration of media within an EMBR. They also reported that the adsorbed TrOC was subsequently degraded by laccase in UF-EMBR, and this prevented the accumulation of TrOCs on the membrane surface. In this experiment, the formation of enzyme gel-layer on membranes surface during EMBR operation was confirmed by characterizing the surface morphology of both the UF and NF membranes by SEM (**Figure 3.10**).

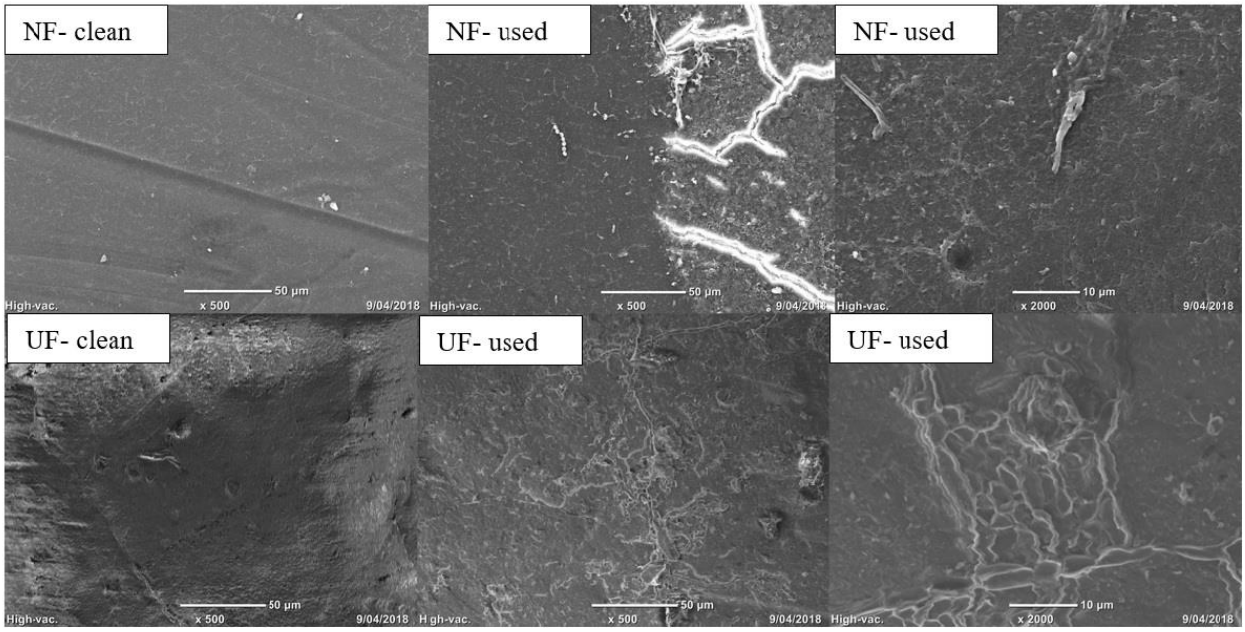

Figure 3.10. SEM images of the NF and UF membranes, confirming the formation of enzyme gel-layer on the surface of membranes. The spiral formations on the SEM images with the annotation 'NF- and UF- used' represents enzyme gel-layer. These spiral formations are not present on the clean NF and UF membrane. The formation of an enzyme gel-layer on the surface of membranes could improve overall performance of EMBRs *via* TrOC adsorption

In a study by Garcia-Ivars et al. [56], partial retention of anionic pharmaceuticals such as naproxen, diclofenac and ibuprofen by a flat-sheet ceramic UF membrane was attributed to charge repulsion mechanism. Similarly, in this experiment, despite being hydrophilic (log D <3), the partial retention of a few anionic TrOCs by the UF membrane was observed. These hydrophilic anionic TrOCs include naproxen (permeate/bioreactor ratio= 0.8), primidone (permeate/bioreactor ratio= 0.86), ibuprofen (permeate/bioreactor ratio= 0.9), propoxur (permeate/bioreactor ratio= 0.88) and diclofenac (permeate/bioreactor ratio= 0.94). Despite the higher MWCO of the UF membrane than the MW of TrOCs, data from the current experiment confirms that the flat-sheet PVDF UF membrane along with the enzyme layer on it can retain anionic TrOCs to some extent *via* charge repulsion mechanism.

The discussion here suggests that UF membrane can contribute to the removal of TrOCs depending on their hydrophobicity and charge, thereby improving the overall performance of UF-EMBR. However, the overall removal by the NF-EMBR was considerably better due to enhanced TrOC degradation (**Figure 3.6**) as well as effective TrOC removal (**Figure 3.8**) in a single step.

Laccase-catalysed degradation of the target pollutants may produce degradation products or metabolites that could be more toxic than the parent pollutants. However, the previous studies suggest that toxicity of EMBR permeate after the enzymatic treatment of a mixture of TrOCs does not increase, particularly when a high retention membrane separation process, *e.g.*, membrane distillation, is integrated with an enzymatic bioreactor [23, 26]. In the current

experiment, a high retention NF membrane combined with the enzymatic bioreactors effectively retained TrOCs (**Figure 3.8**). Thus, the permeate of NF-EMBR was expected to be non-toxic.

3.4.5. Effect of redox-mediator addition on TrOC degradation by NF-EMBR

3.4.5.1. Overall improvement in TrOC degradation

As noted in **section 3.4.3.1**, efficient degradation (>80%) of three non-phenolic and eight phenolic TrOCs was observed during operation of the NF-EMBR (**Figure 3.6**). To improve the spectrum of efficiently degraded TrOCs, redox-mediators can be introduced to the reaction mixture. In a laccase-mediator system, laccase oxidizes the mediator to produce highly reactive radicals. Due to high redox-potential of these radicals, they can directly degrade or polymerize TrOCs, particularly those resistant to laccase-catalyzed degradation [20].

In this experiment, a naturally occurring redox-mediator (violuric acid, VA) was studied for improving the degradation of TrOCs by NF-EMBR.Laccase can readily oxidize VA to form highly reactive aminoxyl (=N–O) radicals. The aminoxyl radicals degrade the target pollutants by following hydrogen atom transfer (HAT) mechanism [25, 57]. The driving force of HAT mechanism is the enthalpy balance between the forming bond (H–ON) and the dissociated C–H bond [20].

Improvement in the degradation of TrOCs following the addition of a single dose of VA at a concentration of 10 μM is presented in **Figure 3.11**. Redox-mediators capable of degrading a substrate following the HAT mechanism has been reported to be particularly effective for non-phenolic compounds, which are originally poorly removed by laccase [25, 58]. In this experiment, VA improved the degradation of six non-phenolic compounds by 10-50% (**Figure 3.11**). For example, diclofenac degradation increased from 13% in NF-EMBR to 42% in laccase-VA mediated NF-EMBR. Similarly, VA addition improved the degradation of the pesticide atrazine by 40%. The highest improvement (50%) was observed for ametryn (**Figure 3.11**). Laccase cannot efficiently degrade non-phenolic TrOCs with higher redox-potential [11, 44]. The redox-potential of the media in enzymatic bioreactor increased from 300 to 390 mV following the addition of VA at a concentration of 10 μM (**Appendix Figure 3-8**), which is one of the reasons of the improved degradation in NF-EMBR. The concentration of redox-mediators is another influencing factor as explained in **Section 3.4.5.2**.

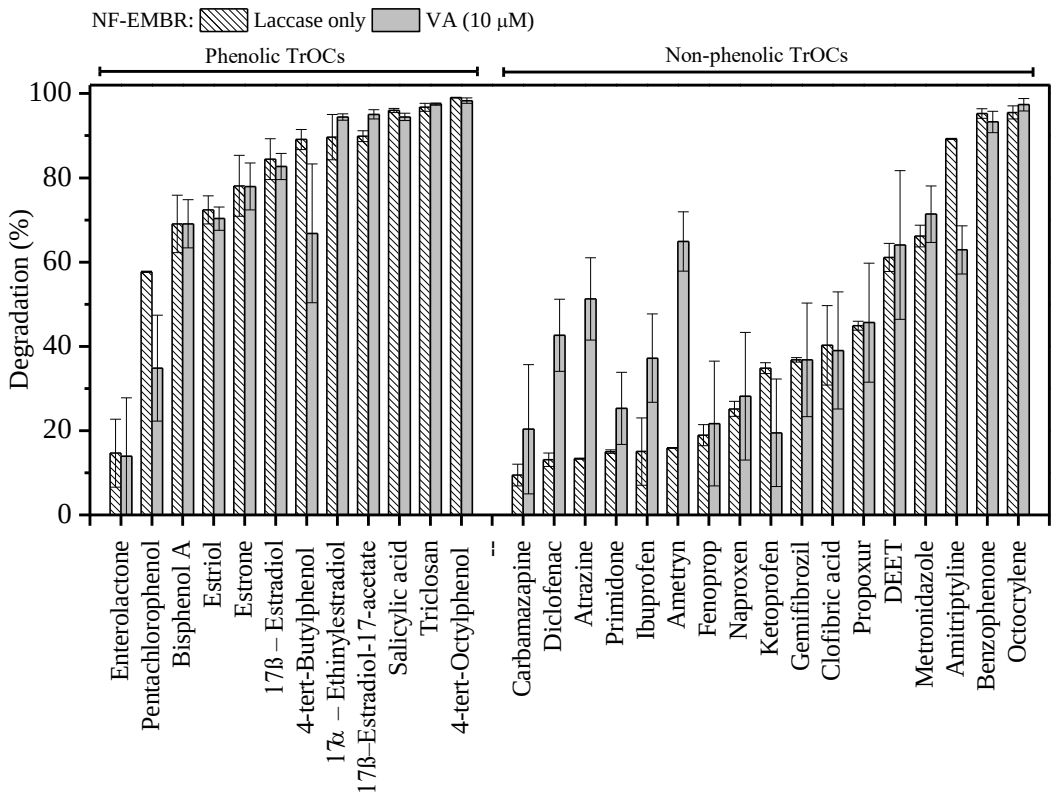

Figure 3.11. Effect of adding a naturally occurring redox-mediator, violuric acid VA, on the degradation of TrOCs in NF-EMBR. VA was added at a concentration of 10 μM at the start of the experiment. Data is presented as the average ± standard deviation (n=4).

Laccase achieved almost complete (>99%) degradation of three phenolic TrOCs *viz* 4-tert-octylphenol, triclosan and salicylic acid in NF-EMBR. However, biodegradation of some phenolic compounds by laccase-only was incomplete. Six steroid hormones (estrone, 17β-estradiol, estriol 17α–ethinylestradiol and 17β-estradiol-17–acetate, enterolactone), two industrial chemicals (4-tert-octylphenol and bisphenol A) and a pesticide (pentachlorophenol) were degraded by laccase with an efficiency between 20 and 90%, and their degradation did not improve at a VA dose of 10 μM (**Figure 3.11**). Our observation is consistent with that by Nguyen et al. [23] who reported that the degradation of phenolic TrOCs such as estrone, estriol, 17β-estradiol 17–acetate, 4-tert-butylphenol and bisphenol A did not improve in UF-EMBR following the addition of another aminoxyl radical producing redox-mediator (*i.e.,* 1-hydroxybenzotriazole, HBT) at a concentration of 10 μM. This is probably because the laccase-VA system did not produce enough reactive aminoxyl radicals at such a trace concentration (*i.e.,* 10 μM) that would improve the degradation of the phenolic TrOCs tested here. Indeed, increasing the concentration of VA from 10 to 25 μM in NF-EMBR resulted in enhanced degradation for six phenolic TrOCs (*see* **Section 3.4.5.2**).

It is noteworthy that redox-mediators have been reported to exhibit substrate specificity [26, 59]. In this experiment, VA (10 μM) was more effective in improving the degradation of the

non-phenolic TrOCs, although it should be noted that the overall degradation of the phenolic compounds within the bioreactor was still significantly better than the non-phenolic TrOCs.

3.4.5.2. Effect of mediator concentration on TrOC degradation

The concentration of redox-mediators can influence the performance of the laccase-mediator system because TrOC degradation is affected by the abundance of highly reactive radicals. Hence, a single dose of VA at different concentrations (*i.e.,* 10, 25, 50 and 100 µM) was added separately to the NF-EMBR. To show different trends of improvement, degradation of 10 selected TrOCs at different VA concentrations is presented in **Figure 3.12**.

Increasing the concentration of VA from 10 to 25 µM further improved the degradation of TrOCs by up to 10-25% (**Figure 3.12**). Although VA did not improve the degradation of phenolic TrOCs at 10 µM, an improvement of 10-25% was observed in the degradation of estrone, estriol, 17β-estradiol 17–acetate, 17β-estradiol, 4-tert-butylphenol and bisphenol A after adding VA at 25 µM concentration in NF-EMBR (**Figure 3.12**). Improvements were also noted in the case of non-phenolic compounds such as propoxur, ibuprofen, diclofenac, ametryn and atrazine. Despite a discernable increase in ORP (*see* **Appendix Figure 3-8**), no further degradation improvement was observed by increasing the concentration of VA from 25 to 100 µM (**Figure 3.12**). Depending on mediator type, laccase source and the target pollutant, the improvement in TrOC degradation may reach a plateau beyond a certain mediator concentration [60, 61]. For instance, Ashe et al. [25] observed no improvement in atrazine and naproxen removal beyond 500 µM of VA in a batch enzymatic bioreactor. In another study, increasing VA concentration from 250 to 500 µM provided similar degradation for a few phenolic TrOCs such as bisphenol A and 4-tert-butylphenol [62]. The current experiment confirms that TrOC degradation would not significantly improve *i.e.,* reach a plateau beyond a certain mediator concentration in a high retention enzymatic bioreactor.

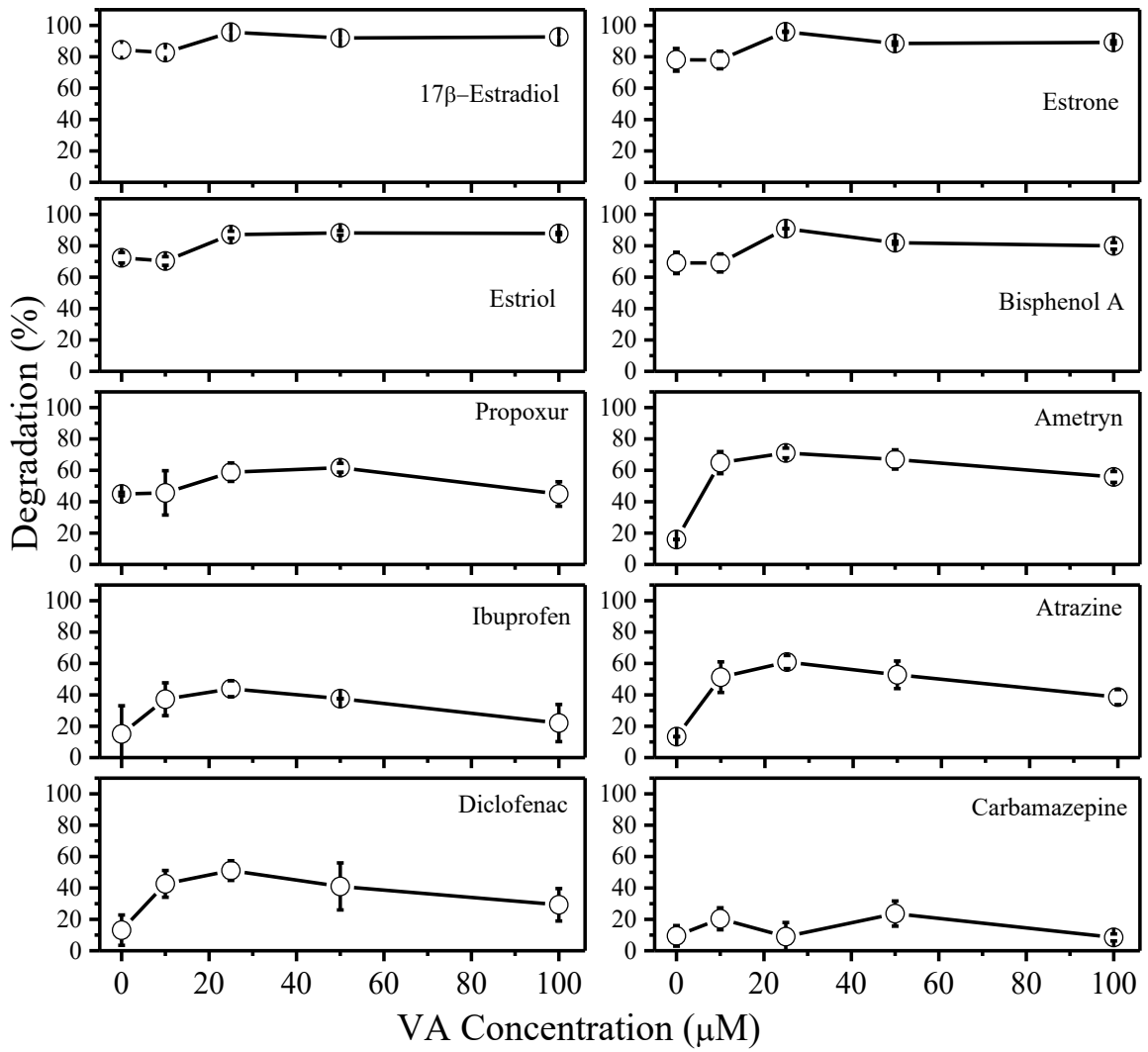

Figure 3.12. Effect of different mediator concentration on the degradation of selected TrOCs in NF-EMBR. Data is presented as the average ± standard deviation (n=4).

Although addiation a redox-mediator improved TrOC degradation, the radicals formed following the oxidation of redox-medaitors can cause toxicity. In prvious studies, addition of syringaldehyde [61] and 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid [25] has been reported to increase the toxicity treated effluent. Notably, following VA addition at concentration ranging from 0.5–1 mM, toxicity of the treated effluent has been reported to not increase significantly [25, 62]. For instance, Asif et al. [62] studied the improvement in TrOC degradation by adding a single dose of 0.5 mM VA in an membrane distillation (MD) – EMBR, and observed that the toxicity of reaction media with and without VA was up to 1.8 and 3.9 rTU, respectivley. Despite the increase in toxicity following the addition of 0.5 mM VA, they reported that the toxicity of MD-EMBR permeate was below the limit of detection [62]. Because a high retention NF membrane that can effectively retained TrOCs and their metabolities was combined with an enzymatic bioreactor in this experiment, permeate of NF-EMBR with and without VA addition was expected to be non-toxic.

In light of mediator performance at different concentrations, VA at a concentration of 25 µM was the best for achieving improved TrOC degradation by the NF-EMBR. Three phenolic and 14 non-phenolic TrOCs were not completely degraded even with redox-mediator dosing. However, the final treated effluent, *i.e*, NF-permeate achieved over 95% removal of all TrOCs (**Figure 3.13**).

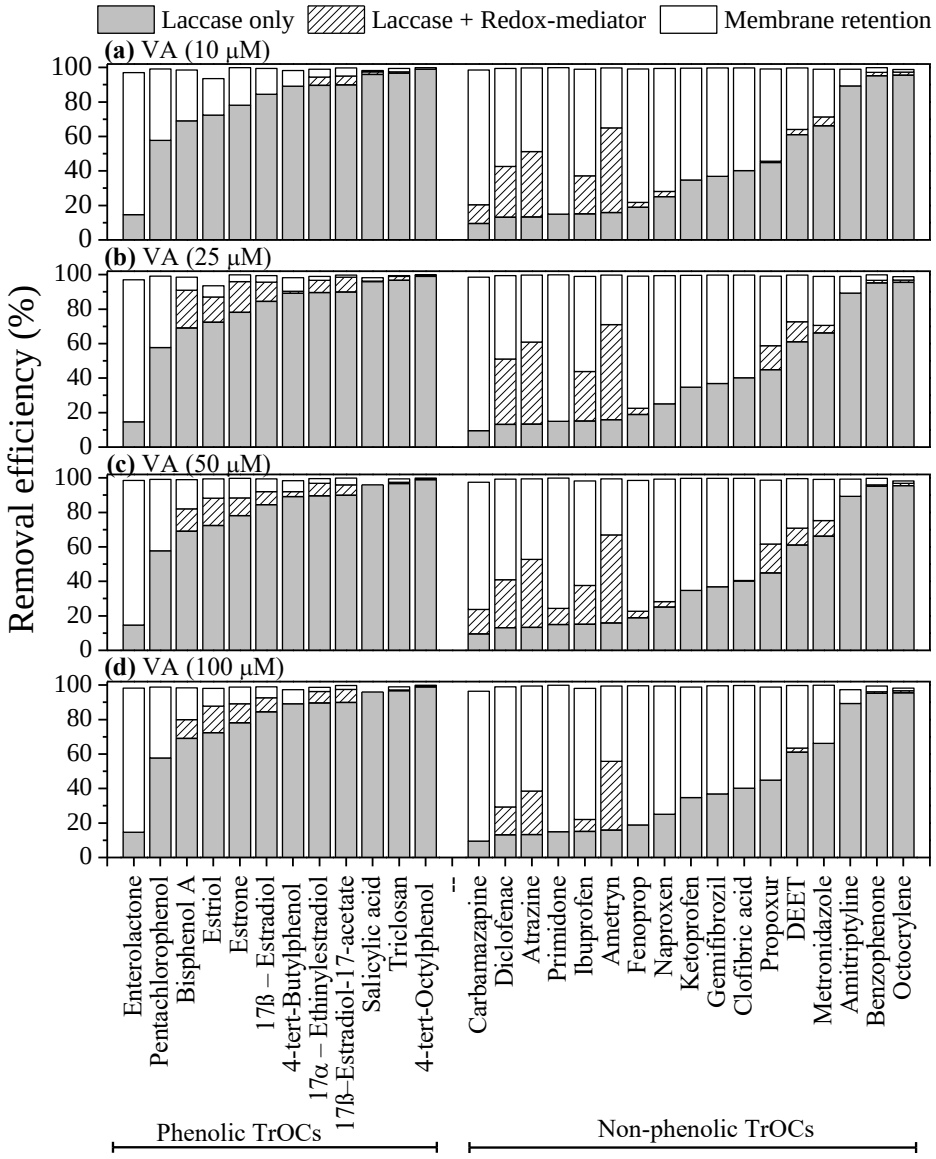

Figure 3.13. Improvement in TrOC degradation by adding single dose VA at different concentration separately at the start of NF-EMBR operation. VA showed compound-specific and concentration dependent improvement. The overall removal of TrOCs in NF-EMBR was >95%. The NF-EMBR were operated for a period of 68 h in continuous mode at an initial HRT of 16 h.

3.4.6. Hydraulic performance of membranes

Variations in permeate flux during the operation of the laccase based EMBRs are presented as normalized permeate flux in **Figure 3.14**. Typically, a steep fall in the permeate flux of the NF and UF membranes has been observed at the initial stage of their operation [24, 56]. Indeed,

the permeate flux reduced rapidly in the first few hours of UF/NF-EMBR runs in this experiment (**Figure 3.14**). Given the MWCO of the membranes, *i.e.*, 200 Da for the NF and 30,000 Da for the UF, the reduction in permeate flux for the NF membrane was steeper. The initial permeate flux of the UF membrane decreased by approximately 15%, and stabilized after 10 h of UF-EMBR operation. On the other hand, a progressive fall in the flux of the NF membrane was observed during the first 30 h of NF-EMBR operation. Despite this, the permeate flux at the end of NF-EMBR operation was still 65% of the initial flux (**Figure 3.14**).

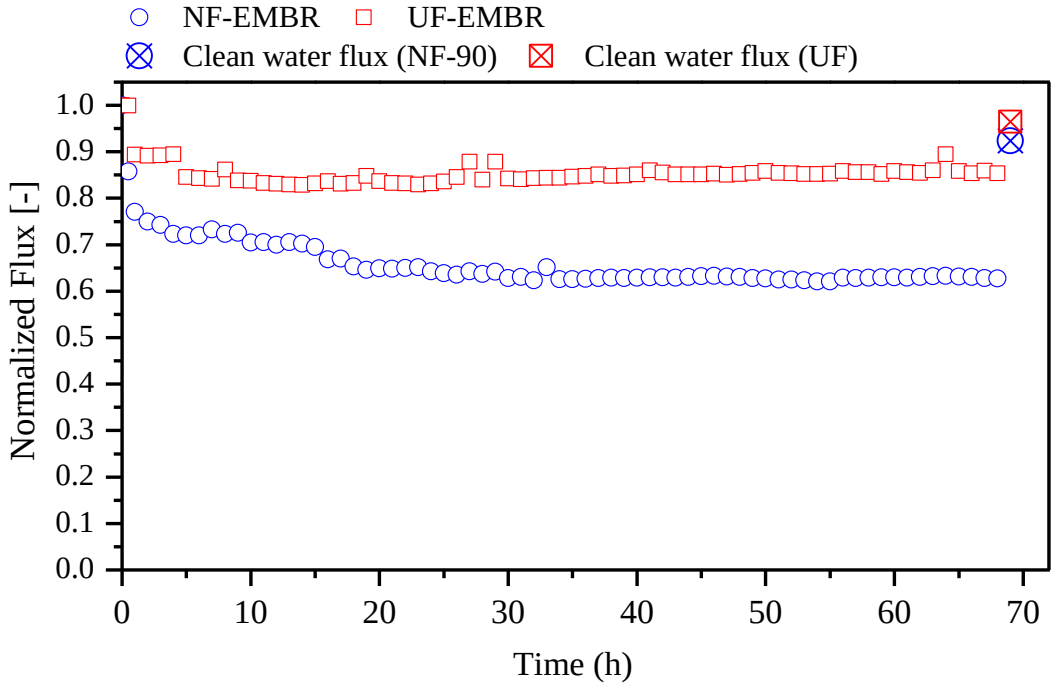

Figure 3.14. Variations in the permeate flux presented as a normalized flux as a function of operating time. The reduction in the permeate flux was attributed to: (i) membrane fouling following the adsorption of laccase on membrane surface forming an enzyme gel-layer (*see* **Figure 3.10**), and/or (ii) concentration polarization due to the accumulation of TrOCs and transformation products on membrane surface. Cleaning the membranes with clean water for one hour was enough to recover the permeate flux by more than 90%.

The reduction in permeate flux in UF/NF-EMBR can be attributed to: (i) membrane fouling due to the adsorption of laccase on membrane surface forming an enzyme gel-layer (**Figure 3.10**); and/or (ii) concentration polarization due to the accumulation of TrOCs and transformation products on membrane surface [24, 63]. To assess whether the reduction in permeate was reversible or irreversible, permeate flux was measured after backwashing the UF and NF membranes with Milli-Q water for 1 h. Membrane cleaning recovered the permeate flux of the NF and UF membranes by 92 and 96%, respectively. The flux recovery was not 100% probably due to the irreversible adsorption of laccase on the membrane surface. This is also evident from changes in membrane properties, *i.e.*, contact angle and zeta potential as discussed in the following section.

3.5. Effect on membrane surface charge and hydrophobicity

The UF and NF membranes were negatively charged at the operating pH of the UF/NF-EMBRs (*i.e.*, approximately 7) as shown in **Figure 3.15**. The virgin NF membrane is negatively charged due to the protonation of carboxylic and amino functional groups of the active membrane layer [34]. On the other hand, the virgin PVDF UF membrane is usually not charged but it becomes negatively charged due to the adsorption of hydroxyl ions that originates from the self-ionization of water [64, 65].

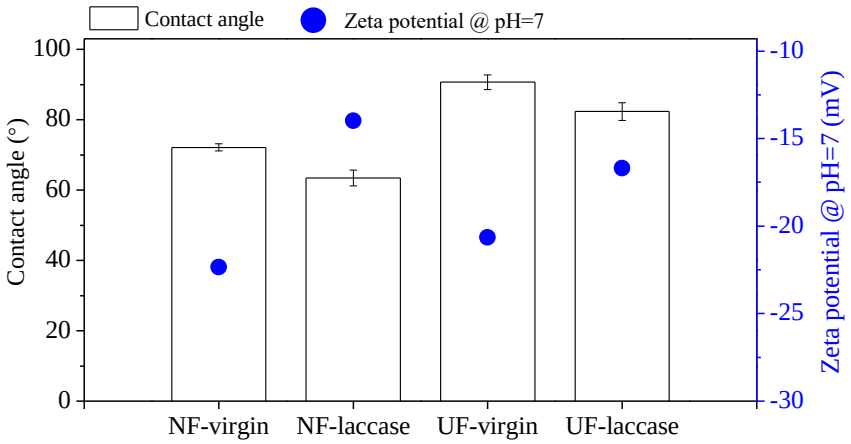

Figure 3.15. Effect of laccase on the properties of the NF and UF membranes. Error bars represent the standard deviation among triplicate measurements. Although change in properties of the NF membrane did not affect TrOC removal by NF-EMBR, the formation of an enzyme-gel layer on the surface of the UF membrane following laccase adsorption can improve the overall performance of UF-EMBR by adsorbing hydrophobic TrOCs (*see* **Figure 3.9**).

The negative charge on the surface of UF and UF membranes in response to their operation with enzyme solution reduced as compared to the virgin membranes (**Figure 3.15**). These changes in membrane surface charge can be attributed to the adsorption of laccase on the membrane surface as shown in **Figure 3.10**. It was reported that adsorption of solutes on the membrane surface can change the surface roughness and chemistry of membrane active layer, thereby altering their streaming potential [66].

Hydrophobicity of a membrane depends on its surface properties and water associating chemical groups [34]. Based on the contact angle, the UF membrane was significantly hydrophobic, while the NF membrane was moderately hydrophobic (**Figure 3.15**). However, hydrophobicity of both the UF and NF membrane reduced, which again confirms the adsorption of laccase on the membrane surface. Results of this experiment indicate that laccase adsorption can alter the properties of the membranes to some extent although above 90% flux recovery can be achieved by flushing the membrane with ultrapure Milli Q water. While no effect of change in properties of the NF membrane was observed on TrOC removal, the formation of an enzyme-gel layer on the surface of the UF membrane following laccase

adsorption can improve the overall performance of UF-EMBR by adsorbing hydrophobic TrOCs (*see* **Section 3.4.3.3**).

3.5. Conclusion

Enzymatic degradation of a broad spectrum of TrOCs including 12 phenolic and 17 non-phenolic compounds was compared by operating ultrafiltration (UF)- and nanofiltration (NF)-based enzymatic membrane bioreactors (EMBR). This helped to assess the effect of effective TrOCs retention within enzymatic bioreactor on their degradation by laccase. Initially, comparative performance of UF- and NF-EMBRs were assessed for five TrOCs to prove the concept. Overall removal of TrOCs by UF-EMBR varied from 20-85%, while NF-EMBR achieved 92-99.9% TrOC removal. Notably, the effective retention of the TrOCs within the enzymatic bioreactor by the NF membrane improved (15-30%) their degradation as compared to UF-EMBR. This observation confirmed the hypothesis that simultaneous retention of laccase and TrOCs by the high retention NF membrane facilitates degradation During the assessment of EMBRs for a broad spectrum of TrOCs, the overall removal of TrOCs in NF-EMBR was better because the NF membrane achieved TrOC rejection ranging from 90-99%. Furthermore, mass balance analysis shows that compared to the UF-EMBR, significantly better degradation (up to 65%) was achieved by laccase in NF-EMBR. However, physciochemical properties, particularly chemical structure governed TrOC removal by laccase and membranes. Laccase achieved efficient degradation of TrOC containing strong electron donating functional groups (such as bisphenol A and natural hormones), while those containing strong EWGs (such as carbamazepine and diclofenac) remained resistant to laccase-catalysed degradation. A redox-mediator (violuric acid, VA) was dosed to NF-EMBR for further improving the degradation of TrOCs. VA achieved improved degradation for four phenolic and six non-phenolic TrOCs in NF-EMBR, at a concentration of 25 µM, beyond which the extent of degradation did not improve significantly. Change in membrane properties due to laccase adsorption along with concentration polarization can reduce the permeate flux of the UF and NF membrane, although flux can be recovered effectively by cleaning the membrane with water.

3.6. References

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Chapter 4: TrOC removal by a membrane distillation – enzymatic membrane bioreactor (MD-EMBR): Impact of laccase source

This chapter is based on the following publication:

Asif, M.B., Nguyen, L.N., Hai, F.I., Price, W.E., Nghiem, L.D. 2017. Integration of an enzymatic bioreactor with membrane distillation for enhanced biodegradation of trace organic contaminants. *International Biodeterioration & Biodegradation, 124, 73-81*.

4.1. Introduction

Trace organic contaminants (TrOCs) include a wide array of natural or anthropogenic chemicals including pesticides, pharmaceuticals and personal care products. Recent studies have confirmed the potentially harmful effects of TrOCs on the growth and reproduction patterns of aquatic flora and fauna as well as on human health due to prolonged ingestion [1, 2]. Conventional wastewater treatment processes cannot effectively remove certain groups of TrOCs, resulting in their widespread occurrence in freshwater sources [3, 4]. Therefore, the scientific community is in constant pursuit of an effective wastewater treatment process for TrOC removal.

Different physicochemical and biological wastewater treatment processes have been investigated over the years for TrOC removal [5-8]. TrOC degradation by biocatalysts such as laccase, peroxidase and proteases is a promising eco-friendly technique [9, 10]. Enzymatic transformation of TrOCs is governed by a number of factors such as pH, temperature, TrOC properties and characteristics of enzymes [11]. Laccase is an oxidase enzyme that can degrade a broad spectrum of TrOCs over a wide range of pH by utilizing the dissolved oxygen in water [12, 13]. Particularly mention worthy is the ability of laccase to oxidize the phenolic TrOCs including aromatic/aliphatic amines, diphenols and methoxy-substituted monophenols [11]. Molecular structure, namely distribution of the functional groups, *i.e.*, electron withdrawing functional group (EWGs) and electron donating functional groups (EDGs), governs the extent of TrOC removal by laccase. The oxidation of TrOCs containing EWGs such as amide ($–NH_2$), halogen (–X) and nitro groups ($– NO_2$) is slower as compared to those containing EDGs [11, 14]. TrOC oxidation can be enhanced by introducing a redox-mediator, which can act as an electron shuttle between the target compounds and enzyme. Depending on the type of redox-mediator, laccase source and TrOC structure, laccase-mediator systems can achieve significant improvement in the removal of target compounds [15, 16]. However, literature on the impact of laccase source and their combination with redox-mediators is limited and require further attention.

Enzyme washout is a major constraint in the large-scale application of an enzymatic bioreactor. To mitigate this problem, laccase can be immobilized onto or entrapped within different supports [11, 17]. Alternatively, enzymatic bioreactor can be coupled with a membrane having a suitable molecular cutoff. For example, Nguyen et al. [13] and Lloret et al. [18] achieved complete retention of laccase with ultrafiltration (UF) membranes. The use of enzymatic membrane bioreactor (EMBR) can avoid the mass transfer limitations associated with laccase immobilization onto support media. Although TrOCs are not expected to be retained by UF membranes, Nguyen et al. [13] observed the formation of an enzyme gel layer on the surface of the membrane that effectively adsorbed non-phenolic hydrophobic TrOCs such as octocrylene, amitriptyline and benzophenone. This resulted in enhanced degradation of these compounds. However, enzyme gel layer could not adsorb hydrophilic non-phenolic TrOCs

such as atrazine and carbamazepine, and their overall removal was less than 10% [13]. Hence, it was postulated that the use of high retention membranes, which will retain both laccase and TrOCs, can facilitate the degradation of resistant TrOCs.

In recent years, high retention membranes, namely membrane distillation [19], nanofiltration [20] and forward osmosis [21-23], have been integrated with the conventional activated sludge bioreactors to achieve complete TrOC retention, resulting in their high aqueous phase removal. However, these short-term studies have revealed accumulation of membrane-retained recalcitrant compounds in the bioreactor, indicating the need for enhancement of biodegradation. Although laccase has been reported to achieve better biodegradation than conventional activated sludge, more efforts are required to explore the performance of high retention membrane – enzymatic bioreactor.

In **Chapter 3**, performance of ultrafiltration (UF) – and nanofiltration (NF) – enzymatic membrane bioreactor (EMBR) operated under identical operating conditions (*e.g.*, hydraulic retention time and TrOC loading rate) is demonstrated. As compared to UF-EMBR, NF-EMBR achieved better degradation of TrOCs. Membrane distillation (MD) is another format of high retention membrane separation process with a completely different working principle and can potentially be integrated with an enzymatic bioreactor for enhanced TrOC degradation. In MD, a vapor-liquid interface is developed around a hydrophobic micro-porous membrane that allows the water to pass through the membrane *via* diffusion due to vapor pressure gradient. A simplified schematic of MD is presented in **Figure 4.1**. Compared to conventional distillation processes such as fractional distillation, the MD process requires low temperature and could be operated by using low grade heat or solar energy [24, 25]. Since the mass transfer in the MD process occurs in gaseous phase, it can theoretically achieve 100% retention of all non-volatile compounds [26]. Previously, the standalone MD process has been investigated for seawater desalination [27], industrial wastewater treatment [28], municipal wastewater treatment [29] and TrOC removal [26].

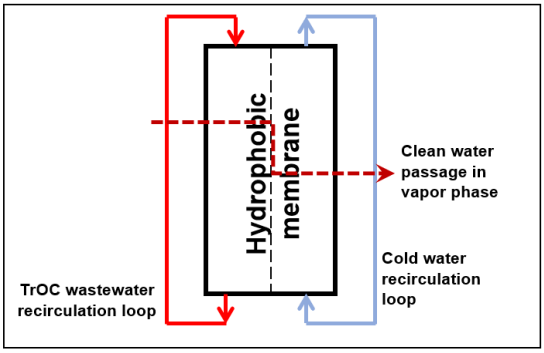


Figure 4.1. A simplified schematic of an MD process. The TrOC wastewater recirculation loop is kept at higher temperature as compared to cold water circulation loop. This creates a vapor

pressure gradient across the MD membrane surface and allows water to diffuse from hot side to cold side.

This chapter is based on the experiment conducted to investigate the performance of a membrane distillation – enzymatic membrane bioreactor (MD-EMBR) for enhanced degradation of five hardly degradable TrOCs. A series of experiments were performed to elucidate the performance of two commercially available laccases – one from genetically modified *Aspergillus oryzae* (*A. oryzae*) and the other from *Trametes versicolor* (*T. versicolor*). In addition, impacts of two N=OH type redox-mediators, namely 1-hydrozybenzotriazole (HBT) and violuric acid (VA) on TrOC degradation as well as on enzyme stability were also studied.

4.2. Hypothesis

- The high retention membrane distillation – enzymatic membrane bioreactor may achieve enhanced TrOC degradation
- Depending on the source of commercially available laccases, the extent of TrOC degradation may be different

4.3. Materials and methods

4.3.1. Trace organic contaminants

Four pharmaceutical and personal care products, namely sulfamethoxazole, carbamazepine, diclofenac and oxybenzone, and one pesticide (atrazine) were selected for this experiment due to their widespread occurrence in environmental systems [4]. Analytical grade (>98% purity) standards of these TrOCs were purchased from Sigma–Aldrich (Australia). The physicochemical properties including molecular weight, chemical structure, hydrophobicity (log D) and volatility (pK_H) of the tested TrOCs are given in **Table 4.1**. A stock solution (2 g/L) of these compounds was prepared and stored at -18 ^oC in the dark.

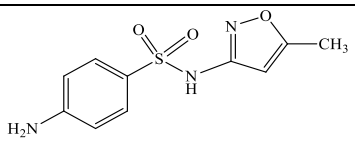
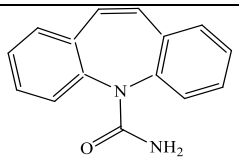
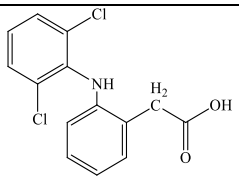
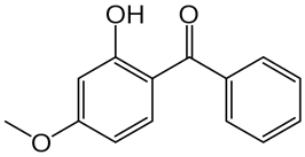
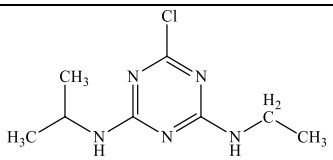
4.3.2. Enzyme solutions and redox-mediators

Commercially available laccase purified from *T. versicolor* (CAS No. 80498-15-3) purchased from Sigma–Aldrich (Australia) was used in this experiment. Laccase from genetically modified *A. oryzae* (Novozym 51030) was the second source of laccase, and it was supplied by Novozymes Pty. Ltd, Australia. These laccases have been investigated recently for the removal of a broad spectrum of TrOCs, showing promising results [13, 15, 30]. Properties of laccase from *A. Oryzae* are already presented in **Section 3.3.1 (Chapter 3).** Laccase from *T. versicolor* was received in powdered form. After dissolving 10 mg of *T. versicolor* laccase in 1 L Milli-Q water, *T. versicolor* laccase showed an activity of 8.5 $\mu M_{(DMP)}$/min at pH 4.5 and 20°C.

Two N=OH type mediators, namely HBT and VA were selected for this experiment. HBT and VA are particularly effective for improving the degradation of non-phenolic compounds. Both

HBT and VA follow hydrogen atom transfer (HAT) mechanism, producing highly stable and reactive aminoxyl radicals [14]. Mediators were also purchased from Sigma–Aldrich (Australia), and separate stock solutions (50 mM) for HBT and VA were kept at 4 ^{o}C before use.

Table 4.1. Physicochemical properties of selected TrOCs

Compounds	Molecular structure	Molecular weight (g/mol)	Log D at pH 7	H (atm m^3/mol)	pK_H at pH 7
Sulfamethoxazole		253.28	-0.22	1.52×10^{-12}	11.81
Carbamazepine		236.27	1.89	8.17×10^{-10}	9.08
Diclofenac		296.15	1.77	2.06×10^{-09}	8.68
Oxybenzone		228.24	3.99	1.58×10^{-08}	7.80
Atrazine		215.68	2.64	5.22×10^{-08}	7.28

Note: Henry's law constant (H) at 25^{o}C (atm m^3/mol) = Vapor pressure × molecular weight/water solubility. The pK_H value is defined as $pK_H = -\log_{10}H$. Chemical structure, molecular weight (MW), log D, vapor pressure and water solubility values were taken from SciFinder Scholar.

4.3.3. MD-EMBR experimental setup

A laboratory scale MD-EMBR setup consisting of a glass enzymatic bioreactor (1.5 L) and an external direct contact membrane distillation (DCMD) module was used (**Figure 4.2**). The enzymatic bioreactor was covered with aluminum foil to avoid TrOC photolysis. An immersion heating unit (Julabo, Germany) was immersed in the water bath to maintain the temperature at

30±0.2 ºC. Moreover, air diffuser connected with an air pump (ACO-002, Zhejiang Sensen Industry Co. Ltd., Zhejiang, China) was placed at the bottom of the bioreactor to maintain homogeneity, and to keep dissolved oxygen (DO) above 3 mg/L.

To minimize heat losses, acrylic glass material was used to prepare the DCMD module. The feed and distillate flow channels (Dimensions: 145 mm×95 mm×3 mm) were engraved on each acrylic block. Water from the enzymatic bioreactor and the distillate container was continuously passed from the DCMD module and then recirculated back to the enzymatic bioreactor and distillate container, respectively. A temperature sensor was placed at the inlet of the DCMD module to monitor the temperature of the feed. Distillate temperature was maintained at 10±0.1 ºC using a chiller (SC100-A10, Thermo Scientific, USA). A stainless-steel heat exchanging coil connected with the chiller was immersed in the distillate container placed on a precision balance (Mettler Toledo Inc, USA) to monitor permeate flux. The recirculation flow rate of both feed and the distillate was kept at 1 L/min (corresponding to the cross-flow velocity of 9 cm/s) using two rotameters.

Hydrophobic microporous polytetrafloroethylene (PTFE) membranes (GE, Minnetonka, MN) were used during all experiments. Properties of PTFE membrane are given elsewhere [19, 31]. Briefly, nominal pore size, thickness, active layer thickness and porosity of the PTFE membrane was 0.22 µm, 175 µm, 70% and 5 µm, respectively.

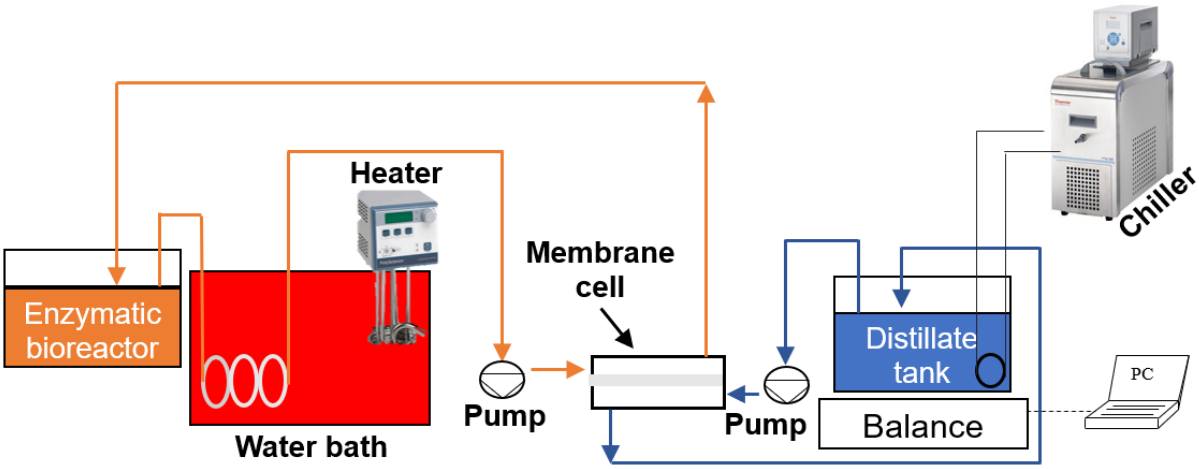


Figure 4.2. A schematics representation of membrane distillation-enzymatic membrane bioreactor (MD-EMBR).

4.3.4. Experimental protocol

Initially, the MD-EMBR system was operated without the addition of enzyme and mediators to determine the loss in TrOC concentrations due to adsorption and/or evaporation. Laccase from *T. versicolor* was tested alone and then both redox-mediators, HBT and VA (at 1 mM concentration), were added separately to investigate the improvement in the degradation of TrOCs. Similarly, laccase from genetically modified *A. oryzae* was also tested with and without

the addition of HBT and VA separately. At the start of each experiment with laccase, 1 mL and 0.1 g of *A. oryzae* and *T. versicolor*, respectively, were added to 1.5 L Milli-Q water separately for achieving an initial enzymatic activity of 95-100 $\mu M_{(DMP)}$/min. TrOCs were each added at a nominal concentration of 1 mg/L. However, the actual measured concentrations of sulfamethoxazole, diclofenac, carbamazepine, atrazine and oxybenzone were 948±90, 923±76, 873±137, 855±140, 771±210 µg/L (n=8), respectively. The difference in theoretical and measured concentrations of TrOCs may be attributed to the purity of each compound, since the actual purity of a compound may differ from that claimed by the manufacturer [32]. Samples from the enzymatic bioreactor and distillate were taken at every three hours over a period of 12 hours to monitor TrOC removal and enzymatic activity. The enzymatic degradation of the TrOCs was determined as $R(\%) = 100 \times (1 - \frac{m_t}{m_0})$, where m_o and m_t are initial mass (0 h) and mass at the time of sampling, respectively.

4.3.5. Analytical methods

4.3.5.1. Analysis of TrOCs

TrOC concentration in the enzymatic bioreactor and permeate was measured at different time intervals using HPLC (Shimadzu, Kyoto, Japan) at the detection wavelength of 280 nm. The HPLC system was equipped with a UV-Vis detector and C-18 column (300×4.6 mm) having a pore size of 5 mm (Supelco Drug Discovery, Sigma Aldrich, Australia). Milli-Q water buffered with 25 mM KH_2PO_4 and HPLC grade acetonitrile were used as the mobile phase for TrOC quantification. Two eluents, namely eluent A (20% acetonitrile + 80% buffer, v/v) and eluent B (80% acetonitrile + 20% buffer, v/v), were passed through the C-18 column at a flow rate of 0.7 mL/min for 30 min in time dependent gradients as follows: [Time (min), A (%)]: [0, 85], [8, 40], [10, 0], [22, 0], [24, 85]. The limit of detection (LOD) for this method was approximately 10 µg/L. Since any residual enzymatic activity in samples may interfere with the accuracy of the results, samples were diluted (2 folds) with methanol to inactivate laccases [15]. Before TrOC analysis, known standards of each TrOC were analyzed to determine the time at which the peak of specific TrOC appears. After that, standards prepared from stock solution containing the mixture of selected TrOCs were analyzed to prepare the calibration curve (peak area *vs* concentration). Coefficient of determination (R^2) for all the calibration curves was greater than 0.99.

4.3.5.2. Laccase assay and ORP

Laccase activity was measured at an interval of three hours using a previously developed method [15]. A detailed description of laccase activity assay and ORP is already given in the **Section 3.3.4.2 (Chapter 3)**.

4.4. Results and discussion

4.4.1. TrOC retention by MD system

TrOC removal by an MD-EMBR system is governed by enzymatic transformation and retention by MD membrane. In this experiment, the MD membrane achieved complete retention (>99%) of the tested TrOCs, *i.e.,* concentration of TrOCs in membrane permeate was below the detection limit of 10 µg/L during all experiments. Since mass transfer in MD occurs in vapor phase, volatility (pK_H) of target pollutants controls their transport from feed to distillate. The retention of TrOCs has been investigated recently in MD-only and MD coupled with conventional bioreactor (MDBR) systems [19, 26], but not an enzymatic bioreactor. In these studies, retention of volatile TrOCs (pK_H <9) by MD system varied from 50-90%, while retention of most non-volatile TrOCs (pK_H >9) varied from 95-99%. Among the incompletely removed moderately-volatile TrOCs in previous studies was oxybenzone [19, 26]. Complete retention of all TrOCs including oxybenzone in the current experiment can be attributed to the lower operating temperature (*i.e.,* 30 ^oC vs. 40 ^oC) of the enzymatic bioreactor, which consequently lowered the vapor pressure.

The MD system was also operated without the addition of laccase to quantify the loss in the mass of tested TrOCs due to adsorption and/or evaporation. Sulfamethoxazole and diclofenac lost approximately 4.5 and 2.5% of its initial mass, respectively, at the end of control run, while the remaining compounds lost less than 1%. A negligible loss in the mass of TrOCs due to adsorption and/or evaporation during the control run suggests that membrane retention and enzymatic degradation were the main mechanisms of TrOC removal in MD-EMBR.

4.4.2. TrOC degradation vs. laccase source in MD-EMBR

Oxidation of TrOCs by laccase is principally controlled by two factors: (i) the nature of functional groups attached to the core part of the molecule *i.e.*, EDGs and EWGs; and (ii) relative redox potential of laccase and TrOCs. Laccase can efficiently degrade phenolic compounds. On the other hand, oxidation of non-phenolic compounds by laccase is possible but it may be restricted by kinetic limitations [11, 14]. Notably, depending on the fungal species, growth medium and level of glycosylation, the catalytic potential of laccase for TrOC removal may be different. Thus, effect of laccase source on TrOC degradation was assessed. In this experiment, significant enzymatic degradation of TrOCs was observed following their complete retention by the MD membrane in MD-EMBR (**Figure 4.3**).

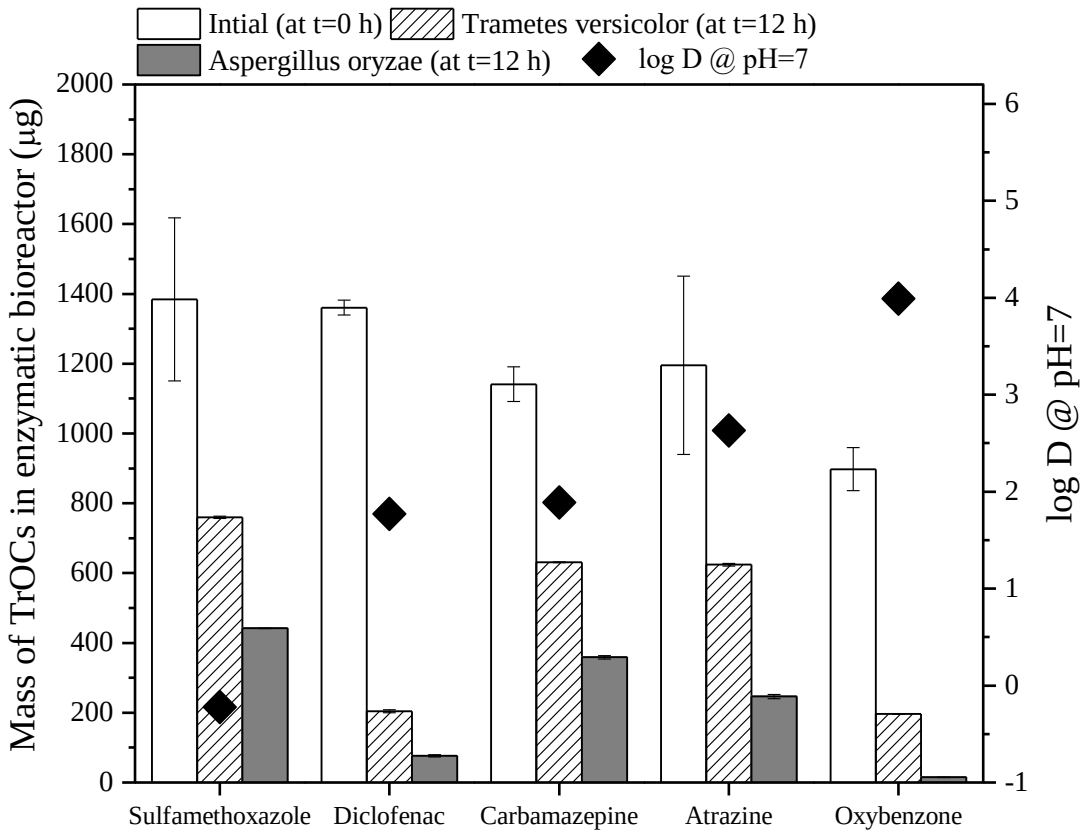


Figure 4.3. Total mass of selected TrOCs in feed at the start (0 h) and the end (12 h) of experiment in the enzymatic bioreactor of MD-EMBR following complete TrOC retention (>99%) by MD. Error bars represent the standard deviation of duplicate samples. Error bars for samples taken at t=12 h are not visible for all the selected TrOCs because the standard deviation among those samples was less than 5%. Operating conditions: temperature of enzymatic bioreactor and distillate was maintained at 30 and 10 °C, respectively; cross-flow rate of water from enzymatic bioreactor and distillate was 1 L/min (corresponding to cross-flow velocity of 9 cm/s); and initial laccase activity in enzymatic bioreactor was 95-100 μM_{DMP}/min.

The fate of each compound was analyzed by developing a mass balance considering the total input mass, mass in concentrate at the end of experiment, mass in permeate, adsorption and/or evaporation losses, and enzymatically degraded portion (**Figure 4.4**). It was observed that biodegradation was the main mechanism of TrOC removal in the enzymatic bioreactor. Laccase from *T. versicolor* and *A. oryzae* achieved 40-80% and 45-99% TrOC degradation, respectively (**Figure 4.4**). Laccase from *A. oryzae* demonstrated better overall performance possibly due to its higher ORP, as discussed further later.

This is the first demonstration of a laccase-based membrane distillation – enzymatic membrane bioreactor (MD-EMBR). Thus, the results are compared with previous UF-EMBR studies to highlight the synergistic effect of integrating a high retention membrane with an enzymatic bioreactor. Given the high TrOC retention by the MD membrane, depending on the level of biodegradation, TrOC concentration in the bioreactor and the MD-permeate may be

significantly different. By contrast, in an UF-EMBR, due to the limited TrOC retention by the cake-layer on UF membrane, the TrOC concentration in the bioreactor and in permeate are usually close *i.e.*, permeate to supernatant ratio is usually within 0.8-1.0 as reported by Nguyen et al. [13]. Therefore, for simplicity we compare overall removal by UF-EMBR (biodegradation plus retention on membrane cake-layer) with biodegradation in MD-EMBR.

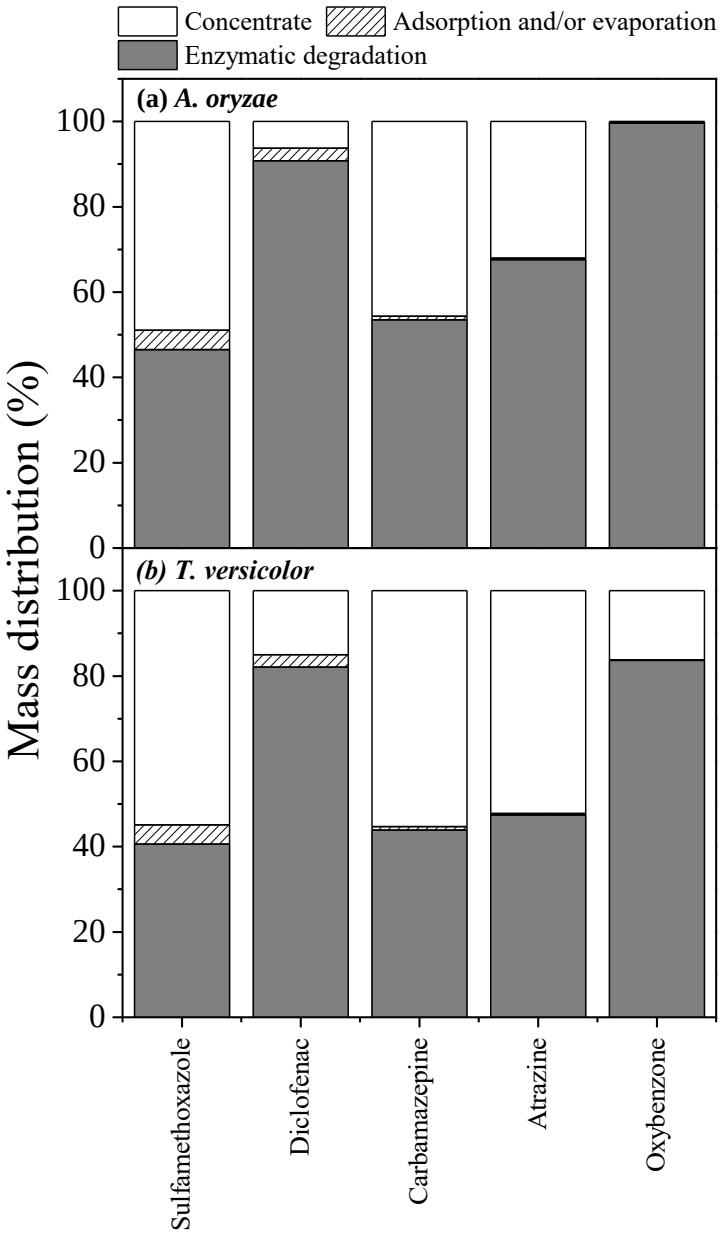


Figure 4.4. The fate of TrOCs following treatment with laccases from *A. oryzae* (a) and *T. versicolor* (b) in the bioreactor of MD-EMBR. The fate of each compound was analyzed by developing a mass balance among the total input, mass in concentrate, enzymatic degradation, adsorption/evaporation and permeates. MD system completely retained (>99%) all the selected TrOCs. Operating conditions of MD-EMBR are given in the caption of **Figure 4.3**.

The highest degradation (80-99%) was achieved for a phenolic compound oxybenzone containing two EDGs, namely methoxy and hydroxyl groups. Previously, Nguyen et al. [13] also observed high overall removal (>80%) of oxybenzone in an UF-EMBR. Conversely, in

comparison to the very low overall removal (>10%) of carbamazepine (containing a strong EWG amide) in previous studies [13, 33], its degradation was 43% with laccase from *T. versicolor* and 53% with laccase *A. oryzae* in the MD-EMBR. In general, diclofenac is well removed (>60%) by laccase in UF-EMBR due to the presence of two EDGs, namely aromatic amine and aniline functional groups, providing active sites for enzymatic attack [13, 18]. In this experiment, degradation of diclofenac by laccases from *T. versicolor and A. oryzae* was observed to be 82 and 90%, respectively.

Despite the presence of two EDGs (methyl and amine functional groups), atrazine, a non-phenolic pesticide, is resistant to laccase based treatment systems due to the steric hindrance caused by chloride, a strong EWG [16, 34]. Enhanced degradation of atrazine by laccases from *T. versicolor* (54%) and *A. oryzae* (67%) compared to its previously reported overall removal of less than 5% in UF-EMBR [13] highlights the importance of complete TrOC retention for efficient enzymatic degradation of recalcitrant TrOCs. Sulfamethoxazole, a significantly hydrophilic compound (log D = -0.22), contains amine (EDG) and sulfonamide (EWG) groups. Depending on the source of fungal laccase and bioreactor type, sulfamethoxazole degradation has been reported to vary significantly (20-80%). For instance, Nguyen et al. [35] achieved less than 20% degradation of sulfamethoxazole by laccase from *A. oryzae* in an UF-EMBR. Conversely, Rodarte-Morales et al. [36] reported around 80% degradation of sulfamethoxazole with laccases from *Bjerkandera sp., Phanerochaete chrysosporium* and *Bjerkandera adusta* in a batch bioreactor. In this experiment, MD-EMBR achieved approximately 40 and 46% degradation of sulfamethoxazole with laccases from *T. versicolor* and *A. oryzae*, respectively.

Integration of a DCMD system with conventional thermophilic bioreactor (MDBR) was investigated by Wijekoon et al. [19]. As expected, the performance of MDBR and MD-EMBR (this experiment) is comparable based on the permeate quality. However, it is indeed important to compare the extent of biodegradation achieved in the bioreactors. The comparison of MD-EMBR with MDBR [19] and UF-EMBR [13, 30] suggests better TrOC removal in the bioreactor of MD-EMBR. For instance, degradation of diclofenac in this experiment ranges from 80-90% (**Figure 4.4**), while MDBR achieved 25% degradation [19]. Similarly, while the conventional activated sludge in MDBR achieved 10% removal of carbamazepine, its enzymatic degradation in MD-EMBR ranged between 43 and 55%.

4.4.3. Effect of redox-mediator addition

4.4.3.1. TrOC degradation

Oxidation of phenolic and non-phenolic compounds by laccase can be possible *via* mono-electronic oxidation subject to their ORP. Low removal of non-phenolic compounds by laccase is due to: (i) their higher ORP than laccase; and/or (ii) steric hindrance caused by EWGs such as chloride and amide functional groups [37]. Several different redox-mediators such as HBT and VA have been studied to improve the ORP of laccase, consequently improving TrOC

degradation [13, 16]. In a laccase-mediator system, laccase reacts with redox-mediators to produce reactive radicals that can improve the effectiveness of laccase-based treatment systems. Moreover, effectiveness of this system depends on mediator type and concentration, chemical structure of the substrate and ORP of laccase [38]. The efficacy of N=OH type mediators for non-phenolic TrOC degradation is evident from literature [16, 37]. Therefore, two N=OH type redox-mediators, namely HBT and VA, at 1 mM concentration were added separately in the enzymatic bioreactor at the start of the experiment. Both mediators follow hydrogen atom transfer (HAT) mechanism and produce highly reactive aminoxyl radical [14].

Regardless of the laccase source, the tested mediators achieved the highest degradation for oxybenzone and diclofenac (**Figure 4.5**), probably because these compounds were already well removed by laccase (**Figure 4.4**). Overall, an improvement of 5-10% in TrOC degradation was achieved in *T. versicolor*-HBT system, while *T. versicolor*-VA yielded 10-20% improvement. Separate addition of HBT and VA with laccase from *A. oryzae* improved the degradation of TrOCs by 12-15 and 15-20%, respectively. Importantly, redox-mediator addition significantly improved carbamazepine and atrazine removal as compared to oxybenzone that was already well removed in the absence of any redox-mediator (**Figure 4.5**).

In line with the results of this experiment, degradation of oxybenzone and diclofenac in the range of 80-99% has been reported following the addition of HBT and VA at 1 mM concentration in batch bioreactors [16, 39]. Similarly, Nguyen et al. [13] achieved 80-85% degradation of oxybenzone following the continuous addition of HBT at a low concentration of 0.01 mM in an UF-EMBR. It also suggests that high concentration (*e.g.*, 1 mM in this experiment) of mediators may not be required to improve the degradation of those TrOCs that are well degraded by laccase.

Improvement in the degradation of non-phenolic compounds has been observed to depend on the type and concentration of redox-mediators [11]. Indeed, improvement in the degradation of non-phenolic TrOCs including carbamazepine, sulfamethoxazole and atrazine was in the range of 10-15 and 15-20% due to the addition of HBT and VA, respectively, (**Figure 4.5**). Based on the overall performance of both laccase sources with VA or HBT (**Figure 4.5**), the laccase from *A. oryzae* again outperformed that from *T. versicolor*.

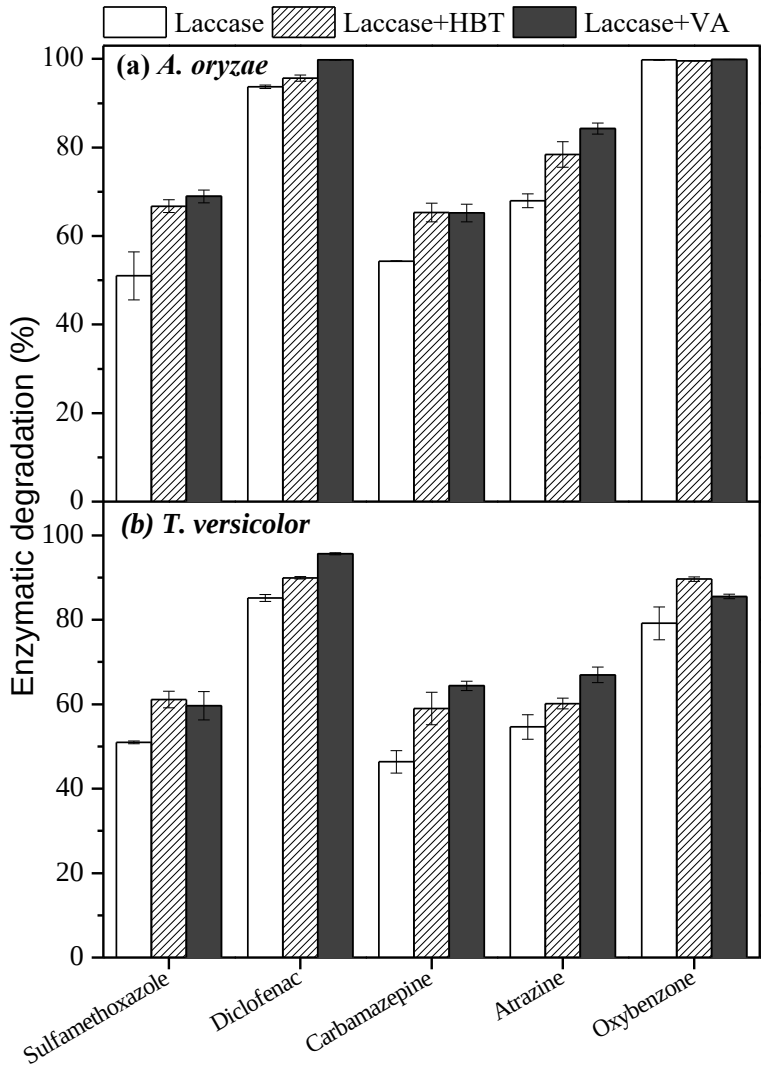


Figure 4.5. Enzymatic degradation of selected TrOCs in enzymatic bioreactor after 12 h of treatment in MD-EMBR with and without the addition of redox-mediator addition. Error bars represent the standard deviation of duplicate samples. Two mediators (HBT and SA) are added separately at 1 mM. Operating conditions of MD-EMBR are given in the caption of **Figure 4.3**.

Increase in the ORP of the reaction media has been suggested as one of the reasons for enhanced TrOC removal in laccase-mediator system [14]. In this experiment, ORP of *A. oryzae* laccase was higher than *T. versicolor* laccase (**Figure 4.6**). Moreover, significant increase in ORP was also observed following the addition of mediators, and its highest value was obtained for VA regardless of the laccase source. Even though ORP of laccase-VA was higher than laccase-HBT, the results (**Figure 4.7**) suggest slightly better degradation of sulfamethoxazole and oxybenzone by laccase-HBT. Therefore, ORP is not the sole factor responsible for enhanced TrOC removal.

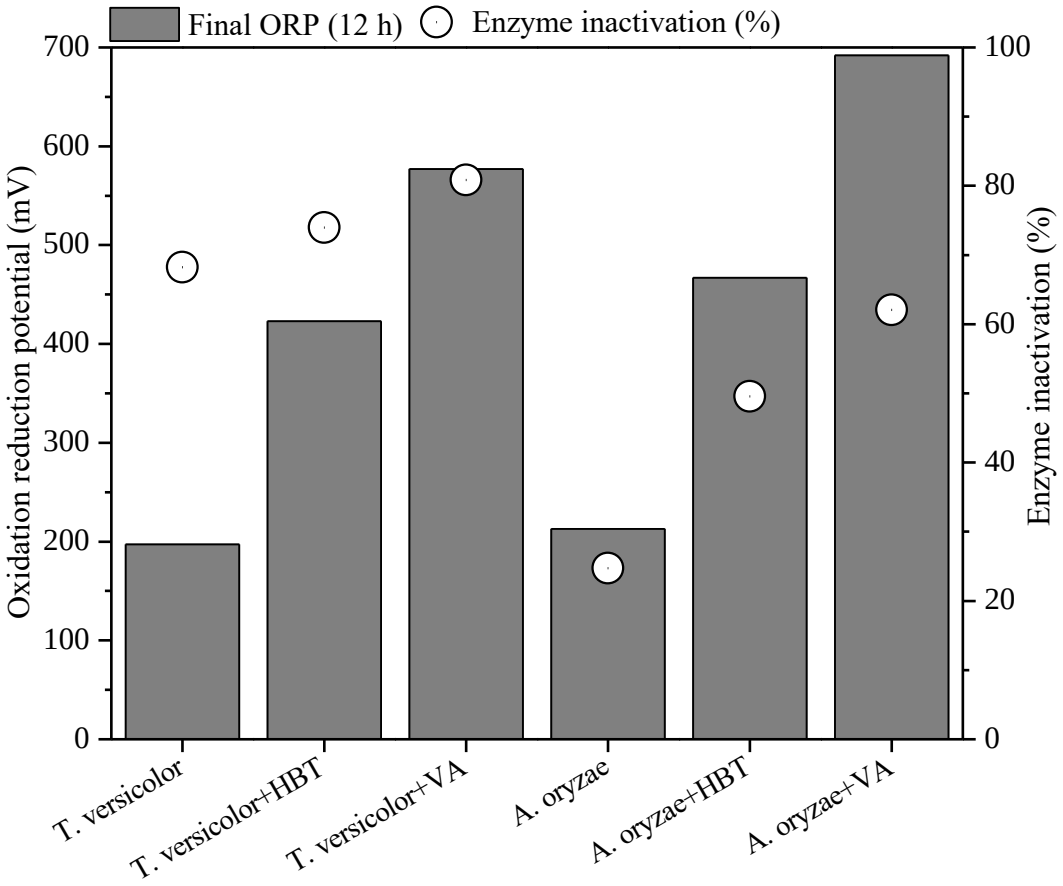


Figure 4.6. Oxidation reduction potential (ORP) and enzyme inactivation with and without the addition of redox-mediators. Two mediators, namely HBT and VA, were added separately at 1 mM concentration. Operating conditions of MD-EMBR are given in the caption of Figure 1. Time course of enzymatic activity during all experiments is given in **Appendix Figure 4-1**.

4.4.3.2. Impact on enzymatic activity

Gradual enzyme denaturation can occur during the operation of an EMBR due to different physical, biological and chemical inhibitors [40]. Moreover, rapid denaturation of enzymes has also been observed following the dosing of redox-mediators [16]. In this experiment, despite the absence of any chemical inhibitor, a continuous drop in enzymatic activity was observed due to hydrodynamic stress during all experiments (*see* **Appendix Figure 4-1**). Enzyme inactivation was significantly increased with the addition of HBT and VA (**Figure 4.6**). Purich [40] suggested that the substrate and charged metabolites can inactivate enzyme in a number of ways such as: (i) substrate can block the active sites of the enzymes due to the electrostatic interactions between enzyme and charged metabolites; and (ii) metabolites can react with enzyme to convert it into nonproductive complexes.

The extent of inactivation in presence of mediators was different for laccase from *T. versicolor* and *A. oryzae*. A direct relation between ORP and enzyme inactivation was observed (**Figure 4.6**). For example, the highest inactivation was induced by VA having the highest ORP (>0.6 V). High ORP of laccase-mediator system indicates that radicals generated due to the oxidation of mediator by laccase can rapidly degrade TrOCs but at the same time they can inactivate

laccase quicker. Therefore, for the development of a long-term laccase-mediator based treatment process, mediator type, concentration and the characteristics of target compounds need to be considered.

4.4.3.3. Impact on contact time

Besides the assessment of the final degradation efficiency at the end of each experiment (*i.e.*, 12 h), TrOC concentration in the enzymatic bioreactor was measured at an interval of three hours. In absence of mediators, regardless of the laccase source, the TrOC concentrations showed a gradual drop over the entire operation period (**Figure 4.7**). On the other hand, in the presence of redox-mediators, the maximum degradation of most TrOCs was achieved within six hours (**Figure 4.7**).

Only oxybenzone degradation was completed within three hours irrespective of redox-mediator addition. Thus, not only that 10-20% improvement in TrOC degradation was achieved (**Figure 4.5**) but that was achieved rapidly (**Figure 4.7**) following the addition of redox-mediators. The cease of TrOC oxidation after six hours was apparently due to the inactivation of the laccase as noted in the previous section. Reactive radicals produced due to the oxidation of redox-mediators by laccase can react with the aromatic amino residues available on the outer surface of the enzyme, resulting in the inactivation of enzyme [41]. Improved TrOC degradation at the expense of high laccase inactivation has been reported previously in batch tests involving laccase-mediator systems [33, 42]. The results presented in this chapter extends such observation in case of an MD-EMBR for the first time.

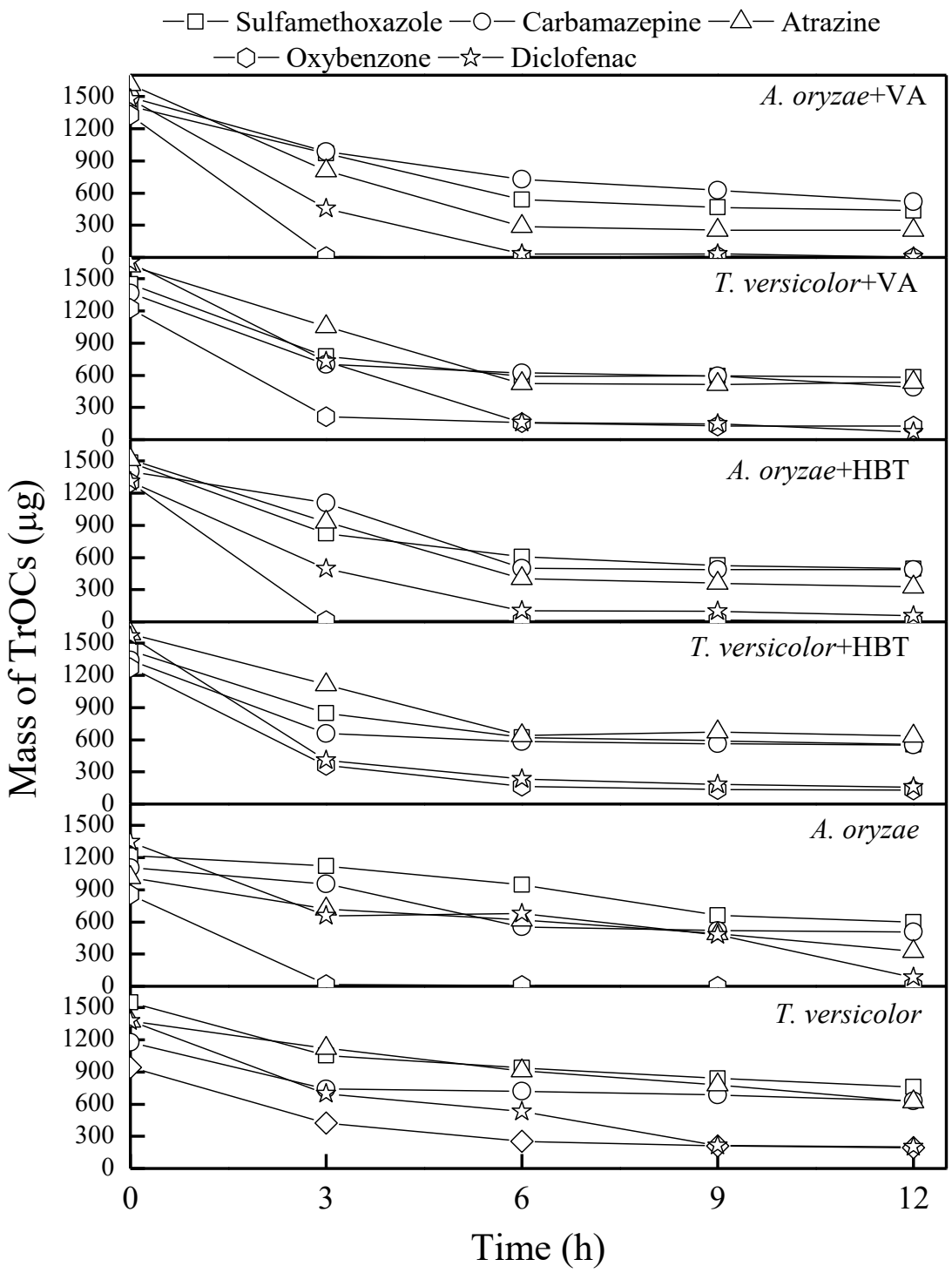


Figure 4.7. Effect of reaction time on the removal of selected TrOCs in the enzymatic bioreactor of MD-EMBR with and without the addition of two mediators. HBT and SA were added at 1 mM concentration separately. Operating conditions of MD-EMBR are given in the caption of **Figure 4.2**.

4.4. Hydraulic performance of membrane

Temperature difference between the feed and the distillate side has major influence over the permeate flux in MD process. Ideally, the temperature at the feed and distillate side is kept at

>50 and 20-25ºC, respectively to produce adequate permeate flux (approximately 10 L/m^2 h) [24]. However, thermal stability of laccase at elevated temperature should be taken in to account before selecting the working temperature of enzymatic bioreactor in MD-EMBR. A few studies have covered the aspect of thermal stability of laccase under different experimental conditions. For instance, Nguyen et al. [43] observed stable laccase activity up to 40 °C when the enzyme solution was not spiked with TrOCs (*i.e.,* 'non-reacting' laccase solution). Conversely, in presence of TrOCs, Nair et al. [44] and Kim and Nicell [45] observed rapid drop in laccase activity beyond 30°C. Therefore, in this experiment, the temperature of the enzymatic bioreactor and permeate was kept at 30 and 10ºC, respectively.

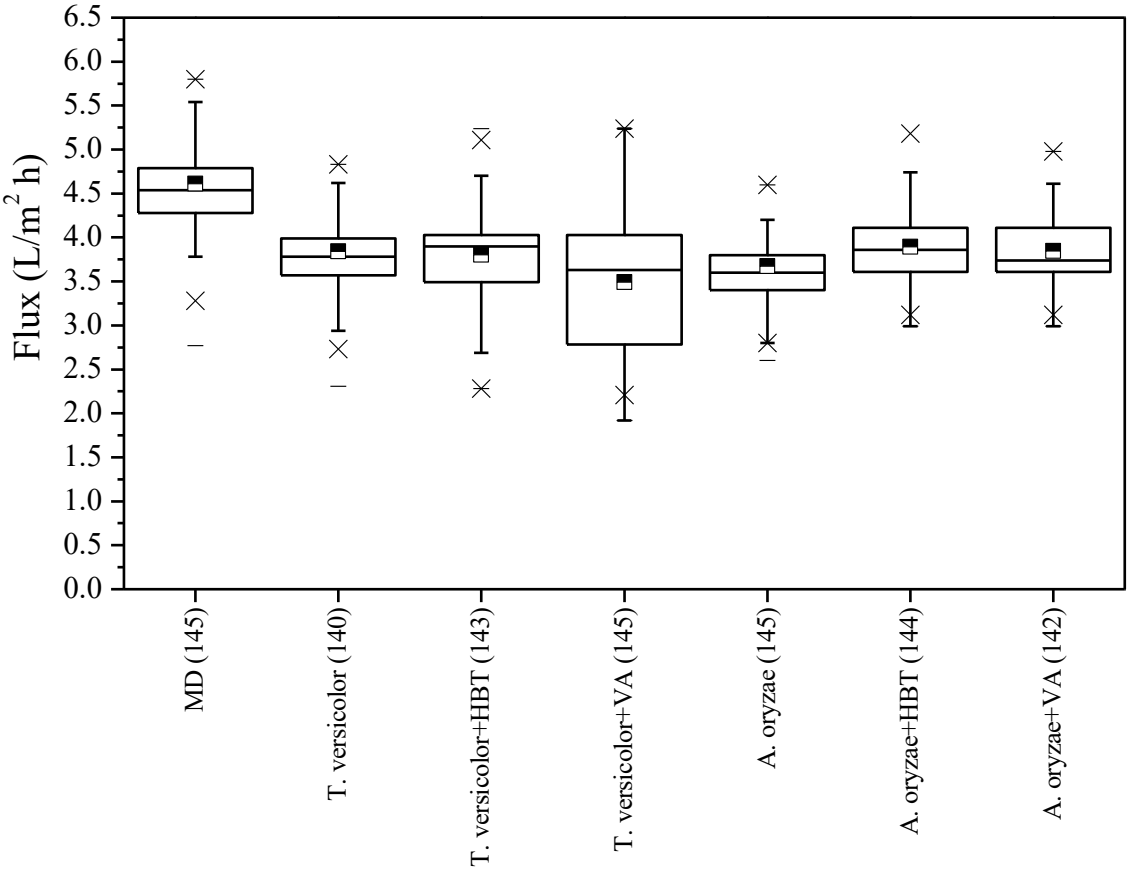


Figure 4.8. Average permeate flux obtained during the operation of enzymatic membrane distillation (E-MD) with different combinations of enzymes and mediators. Numbers within parenthesis in x-axis indicate number of data points. MD without the addition of enzyme and mediators served as a control. Feed and distillate temperature were controlled at 30 and 10 °C, respectively during all experiments. The cross-flow rate of both feed and distillate side was set at 1 L/min (corresponding to a cross-flow velocity of 9 cm/s).

The stability of the permeate flux was continually monitored during all experiments (*see* **Appendix Figure 4-2**). Permeate flux was stable during all experiments, and no significant decline was observed. Average permeate flux of 4.61±0.24, 3.78±0.35 and 3.74±0.46 L/m^2 h was obtained for MD only (control), MD-EMBR with *T. versicolor* and/or mediators and MD-

EMBR with *A. oryzae* and/or mediators, respectively (**Figure 4.8**). Permeate flux depends more on the temperature of the feed side due to the exponential effect of increase in temperature on flux [24]. Thus, relatively low permeate flux in this experiment was expected.

4.5. Conclusion

In this chapter, removal of trace organic contaminants (TrOCs) by an integrated membrane distillation – enzymatic bioreactor (MD-EMBR) was assessed. Experiments were performed using laccase from two different sources, namely *Trametes versicolor* and genetically modified *Aspergillus oryzae* to assess the impact of laccase source on TrOC degradation. Permeate flux of MD-EMBR was stable during all experiments. A mass balance revealed that enzymatic degradation was the major contributor in the overall removal of TrOCs. The MD system ensured complete retention (>99%) of both enzymes and TrOCs. Of particular interest was that the complete retention of the TrOCs resulted in improved TrOC degradation by both laccases. Oxybenzone and diclofenac degradation in the MD-EMBR ranged between 80 and 99%. Compared to UF-EMBR (Section 3.4.2, Chapter 3), up to 40% improvement in the removal of resistant non-phenolic TrOCs (*e.g.,* carbamazepine) was observed. Laccase from *A. oryzae* demonstrated better TrOC degradation and enzymatic stability as compared to laccase from *T. versicolor*. This could be attributed to the higher (15%) redox-potential of laccase from Laccase from *A. oryzae* than laccase from *T. versicolor*. Performance of MD-EMBR system was further improved with the addition of one natural (violuric acid, VA) and one synthetic (1-hydrozybenzotriazole, HBT) redox-mediator at 1 mM concentration. With the addition of redox-mediators, TrOC degradation was improved by 10-20%. Although HBT and VA both affected laccase stability, they increased the reaction rate, which resulted in rapid degradation of the selected compounds.

4.6. References

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Chapter 5: TrOC removal by a membrane distillation – enzymatic membrane bioreactor (MD-EMBR): Impacts of redox-mediator types, concentrations and their mixtures

This chapter is based on the following publications:

Asif, M.B., Hai, F.I., Kang, J., Van De Merwe, J.P., Leusch, F.D., Price, W.E., Nghiem, L.D. 2018. Biocatalytic degradation of pharmaceuticals, personal care products, industrial chemicals, steroid hormones and pesticides in a membrane distillation-enzymatic bioreactor. *Bioresource Technology, 247, 528-536*.

Asif, M.B., Hai, F.I., Kang, J., Van De Merwe, J.P., Leusch, F.D., Yamamoto, K., Price, W.E., Nghiem, L.D. 2017. Degradation of Trace Organic Contaminants by a Membrane Distillation—Enzymatic Bioreactor. *Applied Sciences, 7(9), 879*.

5.1. Introduction

Membrane distillation (MD) is a low temperature distillation process in contrast to conventional distillation processes such as fractional or steam distillation. It essentially relies on the transport of water in the vapor phase from a feed solution through a microporous hydrophobic membrane to the permeate or distillate. Among different MD configurations, direct contact membrane distillation (DCMD) has been predominantly studied due to the ease of its operation [1, 2]. In DCMD, the temperature of the feed solution is maintained at 15-20 ^{o}C higher than the permeate to create an adequate vapor pressure difference, which allows water to pass through a microporous membrane in vapor form *via* diffusion [1, 3]. Since mass transfer occurs in gaseous phase, MD can theoretically achieve complete rejection of all non-volatile compounds [4, 5].

Due to efficient separation efficiency, low fouling propensity and potentially low energy requirement (subject to the availability of low grade heat), stand-alone MD has been studied for applications such as protein recovery in dairy processing [6], treatment of industrial [7] and municipal wastewater [8, 9], as well as for the removal of trace organic contaminant (TrOCs), such as pharmaceuticals and personal care products, pesticides and industrial chemicals, from wastewater [5, 10]. Recently, TrOC removal has also been investigated by coupling an activated sludge based bioreactor to MD that achieved excellent (95-99%) TrOC retention [9]. Since effective retention of TrOCs by the MD theoretically decouples organic retention time from hydraulic retention time (HRT) of a bioreactor, the degradation of TrOCs is expected to improve due to prolonged contact time between the recalcitrant compounds and the microorganisms [11]. However, it was found that the biodegradation of resistant TrOCs, such as those containing strong electron withdrawing functional groups (EWGs), by the activated sludge in the MD-coupled bioreactor did not improve, and eventually these TrOCs accumulated in the bioreactor [9, 12]. Hence, to realize the full potential of a combined biological – MD process, it is necessary to find the means to improve biodegradation of TrOCs retained in the bioreactor by the MD membrane. In this context, it is noteworthy that the oxidoreductase enzyme laccase (EC 1.10.3.2) can degrade TrOCs that are less susceptible to degradation by the activated sludge process [13, 14].

Laccase can catalyze the degradation of a broad spectrum of pollutants including aromatic hydrocarbons, aliphatic amines and TrOCs by using dissolved oxygen as a co-factor [14-16]. TrOC degradation by laccase depends on several factors including pH, temperature, chemical structure of TrOCs and laccase properties [12, 14, 17]. In general, effective laccase-catalyzed degradation of TrOCs containing electron donating functional groups (EDGs) such as amine (–NH_2), alkoxy (–OR) or hydroxyl (–OH) was observed. On the other hand, degradation of TrOCs containing electron withdrawing functional groups (EWGs) such as halogen (–X), amide (–$CONR_2$) or nitro (–NO_2) has been reported to be poor or unstable [14, 18]. Degradation of TrOCs can be improved by adding different natural and synthetic redox-mediators that are

low molecular weight compounds capable of exchanging electrons between laccase and TrOCs [19-21].

Larger scale application of enzymatic treatment systems is restricted by the lack of a bioreactor system, which can prevent washout of enzymes along with treated effluent. In an attempt to prevent enzyme washout, an enzymatic membrane bioreactor (EMBR) was developed by coupling an ultrafiltration (UF) membrane to an enzymatic bioreactor [22, 23]. Interestingly, during the operation of the EMBR, adsorption of some hydrophobic TrOCs (*e.g.*, amitriptyline, oxybenzone and octocrylene) onto the enzyme gel layer over the membrane surface resulted in enhanced degradation of the adsorbed compounds [22]. In another study, removal of four non-phenolic TrOCs, namely atrazine, sulfamethoxazole, diclofenac and carbamazepine were improved by 15–25% following the addition of granular activated carbon (GAC) in EMBR. This was probably because simultaneous adsorption of laccase and TrOCs on GAC promoted the interaction of TrOCs with the active sites of laccase [11]. Results from previous studies indicate the complementarity of simultaneous laccase and TrOC retention within EMBR in contrast to only laccase retention by UF membranes utilized in the previously developed EMBRs [22, 24]. This has led to the development of high retention (HR) – EMBR. Indeed, as demonstrated in **Chapter 3**, TrOC degradation by a high retention nanofiltration (NF) – EMBR was better than those achieved by ultrafiltration (UF)-EMBRs, which retains laccase but not TrOCs. Apparently, the effective retention of the TrOCs by the high retention NF membrane also improved their biodegradation [25].

In a recent study, Asif et al. [24] combined an enzymatic bioreactor with the MD (MD – enzymatic membrane bioreactor or MD-EMBR), which retained both laccase and the tested TrOCs (carbamazepine, sulfamethoxazole, diclofenac, atrazine and oxybenzone). During a short term (12 h) batch operation of the MD-EMBR (*see* **Chapter 4**), degradation of the investigated TrOCs by laccase was found to improve significantly as compared to that achieved by an activated sludge-based MD bioreactor [9, 24]. The initial observations were promising but it is necessary to assess the performance of MD-EMBR for a wide range of TrOCs during continuous operation. In addition, although enhanced degradation was achieved by MD-EMBR [24], degradation of most tested TrOCs was incomplete, requiring the introduction of an additional agent capable of TrOC oxidation. As shown in **Chapter 4**, two redox-mediators, namely 1-hydrozybenzotriazole (HBT) and violuric acid (VA) were introduced separately in the enzymatic bioreactor at a concentration of 1 mM, which improved TrOC degradation by 10-20%. However, there is a need to systematically study the impacts of redox-mediator type and concentration as well as mediator mixtures for improving the degradation of a broad spectrum of TrOCs.

In this chapter, the performance of a laccase-based membrane distillation – enzymatic membrane bioreactor (MD-EMBR) is discussed for the removal of TrOCs having diverse physicochemical properties (*e.g.*, EDGs/EWGs, hydrophobicity and phenolic/non-phenolic

moieties). In addition, the effect of dosing redox-mediators, separately and as a mixture, on TrOC degradation and laccase stability is elucidated. Redox mediators can improve degradation but may increase the toxicity of the treated effluent [26, 27], therefore, the toxicity of the bioreactor media and MD permeate (*i.e.*, final effluent) to bacteria was monitored to clarify the applicability of this treatment process. Finally, during continuous operation, TrOC retention by MD can decrease over time due to 'membrane wetting' or loss of hydrophobicity [1, 3]. Accordingly, the effect of laccase and redox-mediators on the MD performance was also investigated.

5.2. Hypothesis

- Redox-mediator type and concentration may affect the extent of TrOC degradation, enzyme stability
- Mixture of different redox-mediators may achieve better improvement in TrOC degradation than a single redox-mediator

5.3. Materials and methods

5.3.1. TrOCs, laccase and mediators

A synthetic wastewater containing a mixture of 30 TrOCs in Milli-Q water was prepared for this experiment. These compounds were selected to represent different common classes of TrOCs, *viz* pharmaceutical and personal care products, industrial chemicals, steroid hormones and pesticides, which are commonly detected in different environmental systems [28]. A complete list along with their chemical structures appears in **Appendix Table 3-1**. Relevant physicochemical properties of the selected TrOCs such as hydrophobicity (log D) and volatility (pK_H) are given in **Table 5.1**. The list of the selected TrOCs in this chapter and in **Chapter 3** are same except for oxybenzone, *i.e.,* list of TrOCs in Cgapter 3 did not include oxybenzone. Analytical grade TrOCs were purchased from Sigma Aldrich (Australia). A stock solution (25 mg/L) containing the mixture of 30 TrOCs was prepared in methanol and kept in dark at -18 ^{o}C prior to use. Laccase from genetically modified *Aspergillus oryzae* (Novozymes Australia Pty Ltd.) was used. Properties of laccase are already presented in **Section 3.3.1 (Chapter 3)**.

Table 5.1. Physicochemical properties of TrOCs selected for this experiment

TrOCs	Chemical Formula	Molecular Weight	Log D at pH=7	Water Solubility at 25°C	Vapor Pressure	pK_H at pH 7
		g/mole		mg/L	(mmHg)	
Primidone	$C_{12}H_{14}N_2O$	218.25	0.83	1500	6.08×10^{-11}	13.93
Ketoprofen	$C_{16}H_{14}O_3$	254.28	0.19	554,000	3.32×10^{-8}	13.70
Naproxen	$C_{14}H_{14}O_3$	230.26	0.73	435,000	3.01×10^{-7}	12.68
Gemfibrozil	$C_{15}H_{22}O_3$	250.33	2.07	263,000	6.13×10^{-7}	12.11
Metronidazole	$C_6H_9N_3O_3$	171.15	-0.14	29,000	2.67×10^{-7}	11.68
Diclofenac	$C_{14}H_{11}Cl_2NO_2$	296.15	1.77	20,000	1.59×10^{-7}	11.51
Fenoprop	$C_9H_7Cl_3O_3$	269.51	-0.13	230,000	2.13×10^{-6}	11.48
Ibuprofen	$C_{13}H_{18}O_2$	206.28	0.94	928,000	1.39×10^{-4}	10.39
Ametryn	$C_9H_{17}N_5S$	27.33	2.97	140	1.72×10^{-6}	9.35
Clofibric acid	$C_{10}H_{11}ClO_3$	214.65	-1.06	100,000	1.03×10^{-4}	9.54
Carbamazepine	$C_{15}H_{12}N_2O$	236.27	1.89	220	5.78×10^{-7}	9.09
Octocrylene	$C_{24}H_{27}N$	361.48	6.89	0.36	2.56×10^{-9}	8.47
Amitriptyline	$C_{20}H_{23}N$	277.40	2.28	83	1.50×10^{-6}	8.18
Atrazine	$C_8H_{14}ClN_5$	215.68	2.64	69	1.27×10^{-5}	7.28
Propoxur	$C_{11}H_{15}NO_3$	209.24	1.54	800	1.53×10^{-3}	6.28
Benzophenone	$C_{13}H_{10}O$	182.22	3.21	150	8.23×10^{-4}	5.88
DEET	$C_{12}H_{17}NO$	191.3	2.42	1000	5.6×10^{-3}	5.85
Enterolactone	$C_{18}H_{18}O_4$	288.38	2.53	200	3.29×10^{-13}	15.20
Estriol	$C_{18}H_{24}O_3$	298.33	1.89	32	1.34×10^{-9}	10.78
17α–Ethinylestradiol	$C_{20}H_{24}O_2$	269.40	4.11	3.9	3.74×10^{-9}	9.47
Oxybenzone	$C_{14}H_{12}O_3$	228.24	3.89	2700	5.26×10^{-6}	9.23
Estrone	$C_{18}H_{22}O_2$	270.37	3.62	5.9	1.54×10^{-8}	9.03
17β–Estradiol	$C_{18}H_{24}O_2$	272.38	4.15	3	9.82×10^{-9}	8.93
17β-Estradiol-17-acetate	$C_{20}H_{26}O_3$	314.42	5.11	1.9	9.88×10^{-9}	8.67
Bisphenol A	$C_{15}H_{16}O_2$	228.29	3.64	73	5.34×10^{-7}	8.66
Salicylic acid	$C_7H_6O_3$	138.12	-1.13	2240	8.2×10^{-5}	8.18
Pentachlorophenol	C_6HCl_5O	266.34	2.85	4800	3.49×10^{-4}	7.59
Triclosan	$C_{12}H_7Cl_3O_2$	289.54	5.28	19	3.26×10^{-5}	6.18
4-tert-Butylphenol	$C_{10}H_{14}O$	150.22	3.40	1000	0.0361	5.15
4-tert-Octylphenol	$C_{14}H_{22}O$	206.32	5.18	62	1.98×10^{-3}	5.06

Two N=OH type redox-mediators, namely 1-hydroxybenzotriazole (HBT) and violuric acid (VA), and one phenolic redox-mediator, namely syringaldehyde (SA), were used. A separate stock solution (50 mM) of each mediator was prepared in ultrapure Milli-Q water and stored at 4 ºC in the dark. SA and VA produce highly reactive phenoxyl and aminoxyl radicals, respectively. They can mediate TrOC degradation by following a hydrogen atom transfer pathway [16, 27].

5.3.2. The MD-EMBR System

A laboratory scale MD-EMBR system was used comprising a glass enzymatic bioreactor (1.5 L) and an external direct contact membrane distillation (DCMD) module. A schematic of the experimental setup as well as the description of the DCMD module and MD membrane are available in **Section 4.3.3 (Chapter 4)**.

5.3.3. Experimental protocol

5.3.3.1. Preliminary assessment with and without mediator addition

A series of preliminary short-term (12 h) experiments was carried out to evaluate the performance of MD-EMBR for TrOC degradation. At the start of the experiment, a mixture of the selected TrOCs (each at 20 µg/L) in Milli-Q water was added to the bioreactor. Laccase was added to the bioreactor for achieving an initial enzymatic activity of 95–100 $\mu M_{(DMP)}$/min. The media from the glass enzymatic bioreactor and water from the permeate tank were recirculated in their respective flow channels separated by the membrane. A chiller (SC100-A10, Thermo Scientific, Waltham, MA, USA) was used to regulate the temperature of the permeate tank at 10 ± 0.1 °C. The permeate tank was also placed on a precision balance (Mettler Toledo Inc., Columbus, OH, USA) to monitor permeate flux. The recirculation flow rate of both feed and the distillate was controlled at 1 L/min (corresponding to the cross-flow velocity of 9 cm/s) using two rotameters.

Duplicate samples from the enzymatic bioreactor (100 mL each) and permeate tank (500 mL each) were taken after operating the MD-EMBR for 12 h. After evaluating the laccase-catalyzed degradation of TrOCs in MD-EMBR, the possible improvement in TrOC degradation was assessed with the addition of three redox-mediators (HBT, VA and SA) at two different concentrations (0.25 and 0.5 mM) *via* separate runs. Again, duplicate samples from the enzymatic bioreactor and permeate tank were collected for the quantification of TrOCs.

Samples collected from the enzymatic bioreactor were diluted to 500 mL with Milli-Q water and were filtered through 0.45 µm glass fiber filter paper (Filtech, Wollongong, NSW, Australia). The pH of samples was adjusted to 2–2.5 using 4 M H_2SO_4 before solid phase extraction (SPE) and GC/MS analysis. For toxicity analysis, undiluted samples from the enzymatic bioreactor and permeate tank were collected in 2 mL amber vials at the end of each experiment and stored at 4 °C until analysis.

5.3.3.2. Long-term performance with and without mediator addition

A series of long-term experiments were conducted to investigate TrOC retention (by MD membrane) and enzymatic degradation with and without the addition of redox-mediators (*i.e.*, SA and VA) to assess the stability of the developed process. Laccase activity and TrOC concentration in the enzymatic bioreactor were identical to that during preliminary short-term experiments. It is noteworthy that laccase activity in the enzymatic bioreactors may gradually diminish due to various physicochemical and biological inhibitors such as shear stress caused

by membrane filtration [29]. Hence, the laccase activity was maintained at 95-100 $\mu M_{(DMP)}$/min by injecting a small dose of laccase (275 and 400 µL per liter of reactor volume for laccase and laccase-mediator, respectively) every 12 h to sustain MD-EMBR operation.

The MD-EMBR was first operated for a period of 60 h (*i.e.*, 2× HRT) in a continuous mode (*i.e.*, continuous withdrawal of treated effluent) without the addition of mediators. The enzymatic bioreactor was replenished with synthetic wastewater every time the water recovery reached 70% (*i.e.*, approximately around every 24 h). Samples from feed, enzymatic bioreactor and treated effluent (*i.e.*, MD-permeate) were collected after 30 and 60 h of MD-EMBR operation for TrOC quantification. The effect of individual mediators and their mixture on TrOC degradation was investigated in additional runs. A single dose of an individual redox-mediator (at 0.5 mM) or their mixture was added to the enzymatic bioreactor at the beginning of a run. Again, two sets of feed, supernatant and permeate samples for TrOC quantification were collected.

5.3.4. Analytical methods

5.3.4.1. TrOC analysis

TrOCs were analyzed by solid phase extraction (SPE) and quantitative determination by a Shimadzu GC/MS (QP5000) system [9, 30]. A detailed description of this method is given in **Section 3.3.4.1 (Chapter 3)**. The limit of detection (LOD) for this method is compound specific and ranged from 1-20 ng/L as listed in **Appendix Table 3-1**. Removal efficiency by enzymatic bioreactor (R_1) and MD-EMBR (R_2) was calculated as shown in equation (1) and (2), respectively:

$$R_1 = 100 \times (1 - \frac{C_{su}}{C_f}) \quad (1)$$

$$R_2 = 100 \times (1 - \frac{C_p}{C_f}) \quad (2)$$

Where, C_f, C_{su} and C_p are the concentration (ng/L) of a specific TrOC in the feed, supernatant and permeate, respectively. Mass of each TrOC degraded by laccase was calculated as follows:

$$C_f \times V_f = (C_{su} \times V_{su}) + (C_p \times V_p) + \text{mass degraded by laccase} \quad (3)$$

where, V_f, V_{su} and V_p represents the volume of feed, supernatant and permeate, respectively.

5.3.4.2. Laccase activity and contact angle

See **Section 3.3.4.2 and 3.3.4.3 (Chapter 3)**

5.3.4.3. Permeate toxicity analysis

Samples for toxicity analysis were collected from the enzymatic bioreactor and permeate tank at end of each experiment. Toxicity, expressed as a relative toxicity unit (rTU), was analyzed by measuring the inhibition of luminescence in the naturally bioluminescent bacteria,

Photobacterium leiognathi, as previously described [26, 31]. Briefly, an aliquot of a naturally bioluminescent bacteria, Photobacterium leiognathi, was incubated with a serial dilution of the sample extracts in a phosphate buffered saline solution. After 30 min, luminescence was measured on a Fluostar Omega plate reader (BMG Labtech, Germany) and the inhibition of luminescence was calculated relative to a negative control. The IC_{20}, the concentration of the sample required to inhibit bacterial luminescence by 20%, was then computed for each sample by linear regression of the response between 0 and 40% inhibition. All results are presented as a relative toxicity unit (rTU), the reciprocal of the IC20. The limit of detection of this method was 1 rTU.

5.4. Results and discussion

5.4.1. Overall TrOC removal by MD-EMBR

Retention by the MD membrane and degradation in the enzymatic bioreactor are two major mechanisms for TrOC removal in the MD-EMBR system. In theory, MD membranes can retain all but the volatile organic compounds. During the preliminary experiment, the concentration of non-volatile (pK_H > 9; **Table 5.1**) TrOCs in the permeate of the MD-EMBR was below the limit of detection of GC/MS. This is consistent with the observation reported previously, where an MD membrane was coupled with an activated sludge bioreactor [9]. On the other hand, the MD system achieved 90–99% removal (**Figure 5.1**) of relatively volatile TrOCs having pK_H < 9. This compares favorably to their previously reported moderate to high removal (54–99%) by a standalone MD system [5].

In order to assess the stability of the developed process for TrOC removal, the MD-EMBR was operated separately for an extended duration of 60 h (2× HRT). The removal of TrOCs was consistently above 94% and ranged between 94 and above 99% (**Figure 5.1**). Importantly, effective TrOC removal (90->99%) was achieved after the operation of the MD-EMBR for 12 h and 60 h, indicating no deterioration in the quality of membrane permeate. These results suggest that the coupling of enzymatic degradation process to the MD system was favorable for achieving high TrOC removal.

The results obtained after the long-term operation of MD-EMBR indicate that TrOC retention/removal by the MD membrane is not only governed by the vapor pressure (indicated by Henry's constant, H or, pK_H = -log H; **Table 5.1**), but is controlled by both the vapor pressure and the water partition coefficient (log D; **Table 5.1**) of the target TrOC. In a stand-alone MD system, a low (<2.5) 'pK_H /log D' ratio suggests poor removal of the target compound [5]. By contrast, the MD membrane coupled to an activated sludge bioreactor may achieve high removal of the target compounds irrespective of their pK_H /log D ratio. This is because a compound with a low pK_H /log D ratio tends to be adsorbed on the bioreactor particles [9]. Although the enzymatic bioreactor was free of any suspended particles that can potentially adsorb TrOCs, MD-EMBR still achieved 94 to over 99% removal for the 30 TrOCs

tested (**Figure 5.1**). It is noteworthy that compared to their partial removal (54-70%) in a stand-alone MD system [5], the MD membrane in this experiment achieved over 99% removal of some TrOCs including 4-tert-octylphenol (pK_H /log D = 0.98), octocrylene (pK_H /log D = 1.21), 4-tert-butylphenol (pK_H /log D = 1.51), benzophenone (pK_H /log D = 1.83) and oxybenzone (pK_H /log D = 2.1). This significant improvement can be attributed to the efficient degradation of these TrOCs by laccase in MD-EMBR as discussed in the following sections.

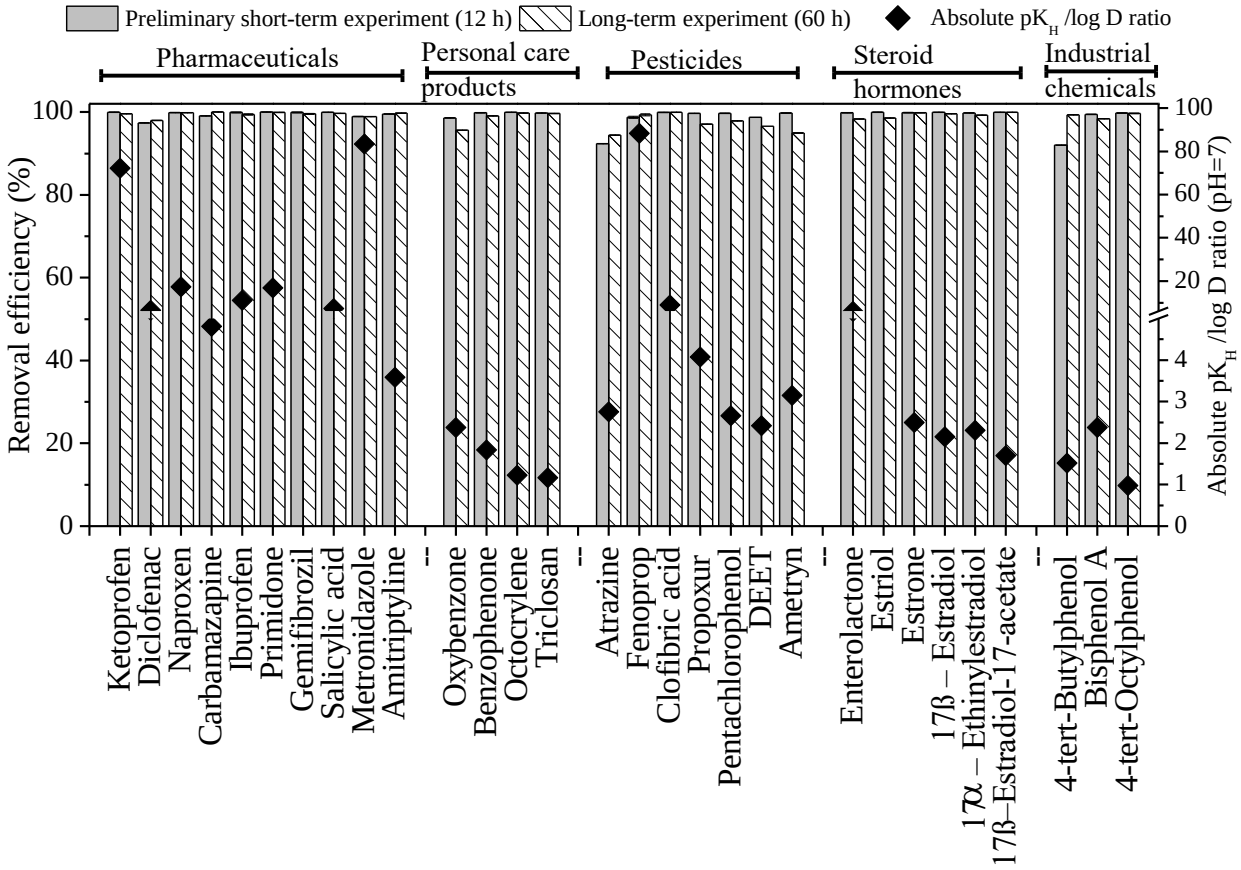

Figure 5.1. Overall removal (membrane retention + enzymatic degradation) of 30 TrOCs arranged by usage category in the MD-EMBR. The data from the preliminary short-term experiment (t = 12 h and n=2) as well as from the long-term experiment (t = 60 h and n=4)) is presented. Error bars are not visible because the standard deviation was less than 5%. MD-EMBR operating conditions: the initial TrOC concentration and laccase activity was 20 µg/L and 95–100 $\mu M_{(DMP)}$/min, respectively; temperature of the enzymatic bioreactor and the permeate tank were kept at 30 and 10 °C, respectively; and cross-flow rate of media from the enzymatic bioreactor and distillate was 1 L/min (corresponding to a cross-flow velocity of 9 cm/s).

5.4.2. Preliminary assessment of TrOC degradation in MD-EMBR

Laccase degrades a substrate *via* a radical-catalyzed mechanism. In this process, transfer of one electron from a substrate to laccase occurs, and molecular oxygen is reduced to water. Laccase can efficiently degrade phenolic pollutants *i.e.,* substrates containing a hydroxyl (–OH) group attached to a benzene ring. On the other hand, non-phenolic pollutants are less amenable to laccase-catalyzed degradation [14, 16]. Therefore, degradation achieved after the preliminary

experiment is presented in **Figure 5.2** by arranging TrOCs based on the presence of phenolic moiety in their molecule.

After the preliminary experiment, high degradation (87–99%) of 10 out 13 phenolic TrOCs was achieved by the MD-EMBR (**Figure 5.2**). These included five steroid hormones (estriol, estrone, 17β–estradiol, 17α–ethinylestradiol and 17β-estradiol-17-acetate (95–99%)), two industrial chemicals (4-tert-butylphenol, and 4-tert-octylphenol (87–99%)) and two personal care products (oxybenzone and triclosan (89–98%)). On the other hand, enzymatic degradation of some phenolic compounds, namely pentachlorophenol, enterolactone and salicylic acid, ranged from 55 to 75%. The enzymatic degradation of 17 non-phenolic TrOCs varied from 40 to 99% (**Figure 5.2**). Laccase-catalyzed degradation of 13 compounds fell in the range of 40–65%, while the degradation of the remaining four non-phenolic TrOCs ranged between 94 and 98%. The well degraded non-phenolic TrOCs include metronidazole, benzophenone, amitriptyline and octocrylene. High laccase-catalyzed degradation (80–99%) in continuous flow UF-EMBR has been previously reported [22, 26] for benzophenone, amitriptyline and octocrylene.

An overall degradation of only 40–65% was achieved by the MD-EMBR for a number of non-phenolic TrOCs (**Figure 5.2**), however, these removal efficiencies in fact compare favorably with those reported in the literature [17, 19, 26]. For instance, laccase-catalyzed degradation of carbamazepine, clofibric acid, fenoprop and atrazine has been reported to be less than 10% in both batch and continuous-flow ultrafiltration based enzymatic bioreactors [19, 22, 32]. By contrast, 40–45% degradation of these TrOCs by the MD-EMBR was observed during preliminary assessment. Importantly, degradation of TrOCs by laccase in the MD-EMBR seems to be governed by TrOC properties such as the presence of strong EDGs and/or EWGs. This has been explained comprehensively in **Section 5.4.2.2** that elucidates TrOC degradation in the MD-EMBR during long-term operation.

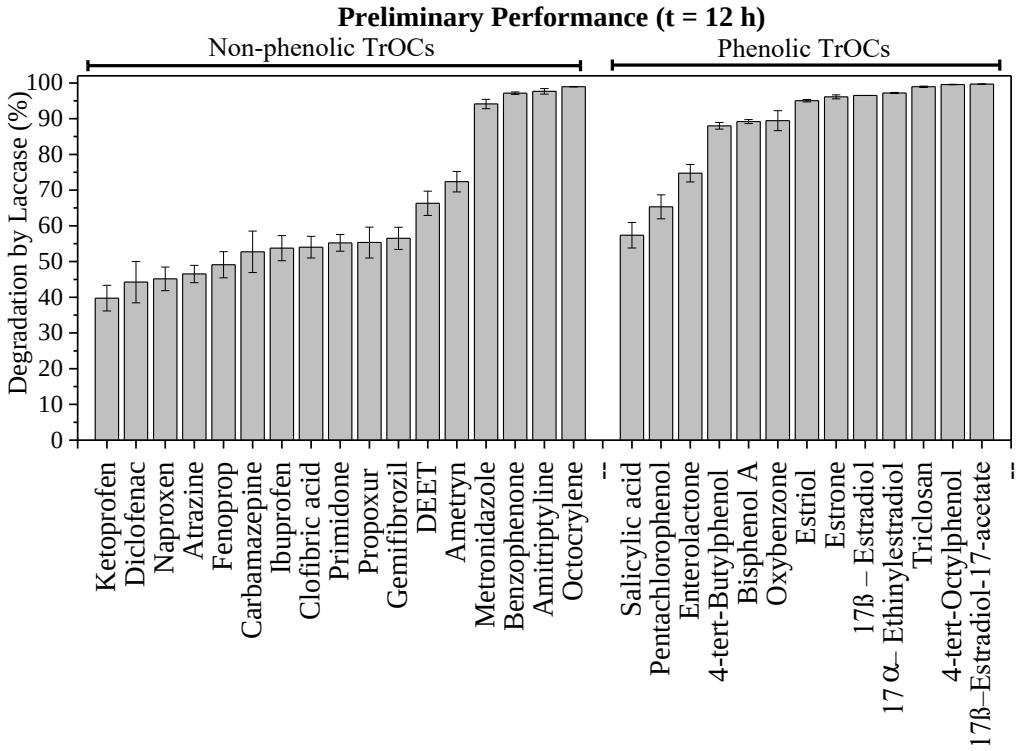

Figure 5.2. Preliminary performance of the MD-EMBR for the degradation of 30 TrOCs by laccase. Error bars indicate the standard deviation of duplicate samples. Experimental conditions are given in the caption of **Figure 5.1**.

5.4.3. TrOC degradation in MD-EMBR during long-term continuous operation

5.4.3.1. Degradation of phenolic TrOCs

Of the 13 phenolic TrOCs tested (**Figure 5.3**), laccase achieved significant degradation (95-99%) of 10 compounds including five steroid hormones, three industrial chemicals (bisphenol A, 4-tert-butylphenol and 4-tert-octylphenol) and two ingredients of personal care products (triclosan and oxybenzone). The observation of efficient enzymatic degradation of these TrOCs in MD-EMBR is consistent with the literature regarding previously developed enzymatic bioreactors. For example, Lloret et al. [23] achieved 95-99% removal of two steroid hormones (estrone and 17β–estradiol) in a batch enzymatic bioreactor. Similarly, efficient degradation (>90%) of oxybenzone, bisphenol A, triclosan and 4-tert-butylphenol has been achieved by both batch or continuous-flow enzymatic bioreactors [17, 26]. Notably, a reduced removal (20-35%) of three natural steroid hormones such as estrone, 17β-estradiol and estriol has been reported in continuous -low UF-EMBRs, as compared to that achieved by batch enzymatic bioreactor [22, 23]. This was attributed to the sustained-TrOC loading in UF-EMBRs. In this experiment, degradation of estrone, 17β-estradiol and estriol was greater than 99%, which indicates that effective retention of these TrOCs by the MD membrane facilitated their degradation by laccase in MD-EMBR.

Although phenolic TrOCs are especially amenable to laccase-catalyzed degradation, moderate degradation of a few phenolic compounds has been previously attributed to the presence of

EWG(s) in their molecular structure [33]. Due to the steric hindrance caused by the concomitant presence of an EWG, phenolic TrOCs cannot access the active sites of laccase for efficient degradation [26, 33]. In line with this, a moderate degradation (44-65%) was observed for three phenolic TrOCs, namely salicylic acid, pentachlorophenol and enterolactone in this experiment, which contain an EWG (*i.e.*, carbonyl or halogen) in their molecule (**Figure 5.3**).

5.4.3.2. Degradation of non-phenolic TrOCs

Laccase can oxidize non-phenolic TrOCs, but the extent of the degradation may not be significant [14]. In previous studies, two distinct trends were observed for the degradation of non-phenolic TrOCs by laccase: (i) poor removal (*e.g.*, less than 5%) of those that only contain strong EWGs such as halogen (–X), amide ($–CONR_2$) and carbonyl (–C=O) functional groups; and (ii) moderate to high removal of those that contain both EWGs and EDGs such as amine ($–NH_2$) or alkoxy (–OR) functional group [14, 29, 34]. In this experiment, benzophenone, octocrylene and amitriptyline were significantly degraded (>95%) by laccase. On the other hand, a moderate degradation (45-75%) was observed for the remaining non-phenolic TrOCs (**Figure 5.3**).

Of particular interest is the enhanced degradation of pharmaceuticals and pesticides (containing strong EWGs) that were previously reported to be poorly (<10%) degraded by laccase in both batch and continuous-flow enzymatic bioreactors [17, 26]. These TrOCs include ketoprofen (EWG carboxylic; 52% removal), clofibric acid (EWG halogen; 55% removal), carbamazepine (EWG amide; 62% removal), metronidazole (EWG nitro; 67% removal), atrazine (EWG halogen; 59% removal), fenoprop (EWG halogen, 48% removal) and N, N-Diethyl-meta-toluamide (DEET; EWG amide; 69% removal) (**Figure 5.3**). Previously, significantly improved degradation of recalcitrant TrOCs such as carbamazepine, atrazine and diclofenac was attributed to simultaneous adsorption of laccase and TrOCs on granular activated carbon which allowed prolonged close contact between laccase and TrOCs [11]. Although approach in this experiment was different, the enhanced degradation of recalcitrant TrOCs in MD-EMBR can be ascribed to the increased contact time between laccase and TrOCs following their complete retention (95-99% removal) by the MD membrane. It is important to critically analyze the degradation of TrOCs after both 12 h (preliminary performance) and 60 h experiments (long-term performance). The comparison of TrOC degradation by the MD-EMBR after 12 h (**Figure 5.2**) and 60 h (**Figure 5.3**) suggests that TrOC degradation was stable.

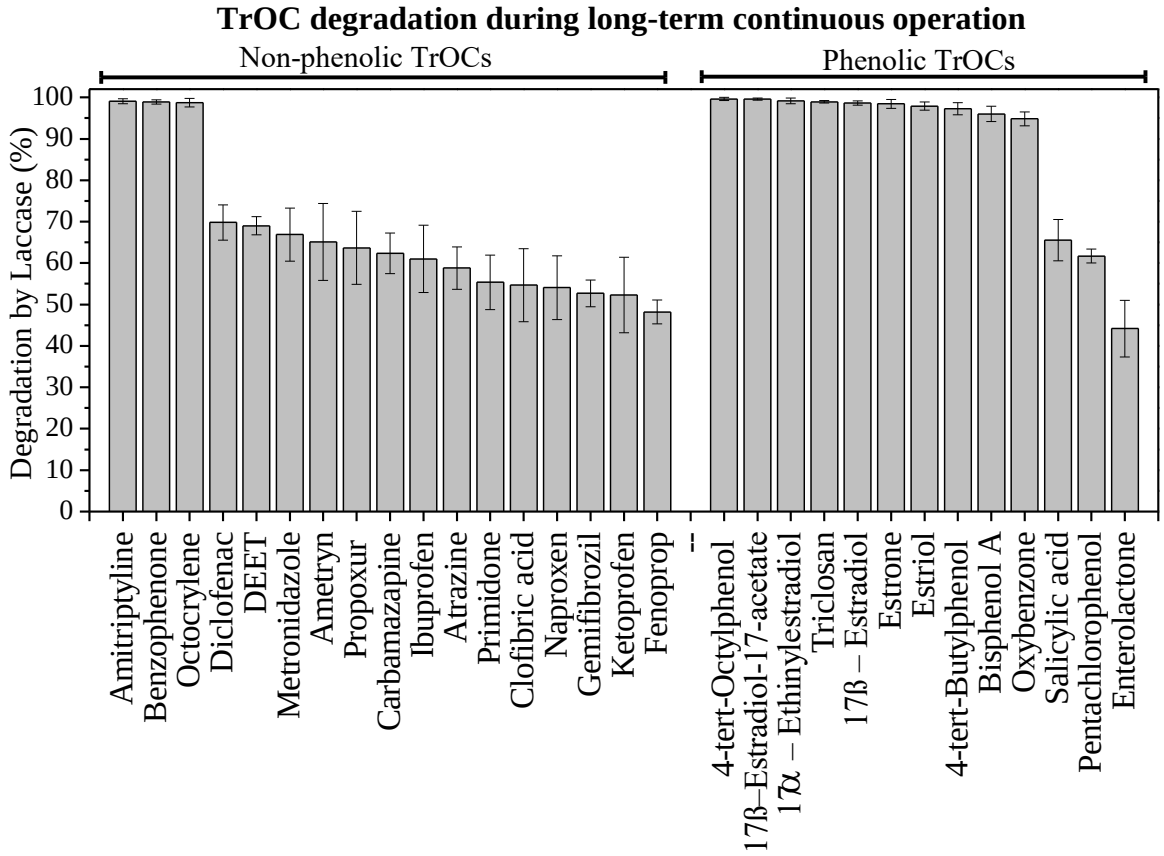

Figure 5.3. Laccase-catalyzed degradation of 30 TrOCs in MD-EMBR during long-term continuous operation of MD-EMBR (60 h; and 2×HRT). Error bars indicate the standard deviation among four samples. Experimental conditions are given in the caption of **Figure 5.1**.

It is also noteworthy that TrOCs containing EDGs such as hydroxyl and amine (*e.g.,* steroid hormones, bisphenol A and triclosan) can act as bi-functional substrates or redox-mediators [33, 35]. Fragments of phenoxyl radicals or oxidative coupling agents (*e.g.,* dimers) produced due to the oxidation of bi-functional substrates can facilitate the degradation of recalcitrant TrOCs *via* enzymatic and/or non-enzymatic reactions (*e.g.,* polymerization or agglomeration). Indeed, Margot et al. [17] reported significantly higher diclofenac removal in presence of the phenolic TrOC bisphenol A as compared to that observed for diclofenac as a single compound. Similarly, Nair et al. [36] observed above 90% removal of diclofenac in a mixture containing bisphenol A, 17α–ethinylestradiol and diclofenac as compared to its 70% removal in absence of the phenolic TrOCs. In another study, Hachi et al. [35] demonstrated that an oxidative coupling agent (*i.e.,* dimer) produced due to the degradation of acetaminophen containing an EDG (*i.e.,* amine) formed oligomers with carbamazepine. These oligomers were more susceptible to laccase-catalyzed oxidation than the parent compound, which led to enhanced carbamazepine degradation [35]. Furthermore, in nature, laccase oxidizes the aromatic rings of lignin and produce phenoxyl radical, which are responsible for the degradation of non-phenolic components of lignin [33, 37]. Thus, there is a strong body of evidence of TrOCs containing EDGs working as redox-mediators for enhancing degradation of non-phenolics, albeit from

batch tests only. The synthetic wastewater used in this experiment contained a mixture of TrOCs containing EDGs and EWGs, as would be expected in practical wastewater conditions. These TrOCs were well retained by the MD component during the continuous operation of the MD-EMBR (*see* **Section 5.4.1**). Therefore, it is possible that radicals or oxidative coupling agents formed due to the oxidation of TrOCs containing hydroxyl and amine functional groups also contributed in achieving enhanced degradation (as compared to that achieved by UF-EMBRs) of resistant TrOCs containing EWGs by the MD-EMBR. A comparative performance of a high retention – and conventional EMBR is demonstrated in **Chapter 3**.

5.4.4. MD-EMBR performance with mediator addition

5.4.4.1. Preliminary screening of redox-mediators during preliminary assessment

As noted in **Section 5.4.2** (*i.e.*, preliminary assessment of TrOC degradation in MD-EMBR), of the 30 TrOCs tested, MD-EMBR achieved high degradation (85–99%) for 14 compounds (10 phenolic and 4 non-phenolic compounds) but the degradation efficiency varied widely (40–70%) for the rest of the compounds. To improve the degradation of the latter group, three redox-mediators, namely SA, VA and HBT, were added at 0.25 and 0.5 mM concentrations each in separate runs. Depending on the redox-mediator type and concentration, degradation of phenolic compounds and non-phenolic compounds by the MD-EMBR was improved by 20–30% and 10–50%, respectively (**Figure 5.4**) as explained below.

To date, the impact of redox-mediator type on the improvement of TrOC degradation has been assessed mainly in small scale and batch tests [27, 38, 39]. For instance, Ashe et al. [27] investigated the performance of seven different redox-mediators including SA, HBT and VA for the degradation of four resistant TrOCs, namely atrazine, naproxen, oxybenzone and pentachlorophenol in 10 mL batch reactors. They achieved significant improvement (40–90%) at a concentration of 1 mM. Nguyen et al. [22] achieved enhanced (10–90%) removal of TrOCs in UF-EMBR using SA and HBT. However, this is the first experiment investigating the efficacy of SA, VA and HBT for enhanced degradation of a broad spectrum of TrOCs by an MD-EMBR.

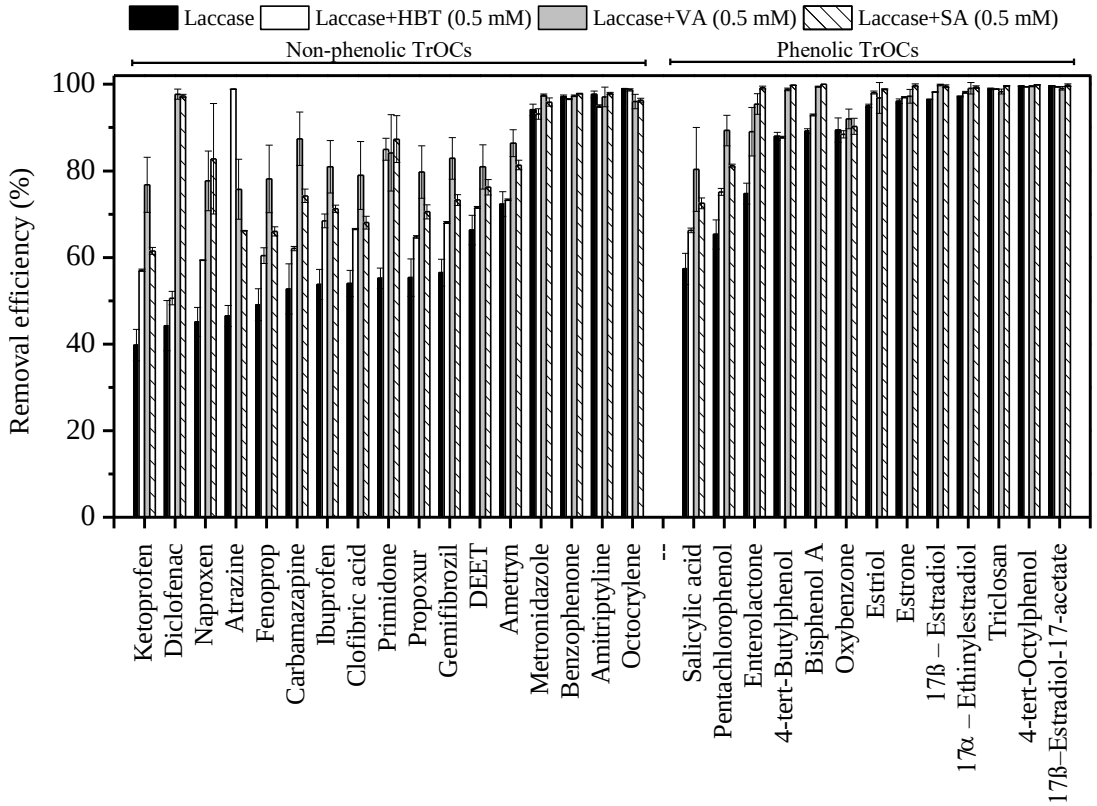

Figure 5.4. Enzymatic degradation of 30 TrOCs during the preliminary screening of three redox-mediators, namely HBT, VA and SA (separately at 0.5 mM) in the MD-EMBR operated for 12 h. Error bars indicate the standard deviation of duplicate samples. Operating conditions of the MD-EMBR are given in the caption of **Figure 5.1**.

All the tested redox-mediators enhanced the degradation of TrOCs. However, the best overall performance was shown by VA (**Figure 5.4**). In line with the findings of Nguyen et al. [26], degradation of the phenolic TrOCs that were already highly degraded by laccase (**Figure 5.2**) remained almost the same after the addition of redox-mediators. For the remaining phenolic TrOCs, VA (at 0.5 mM), compared to HBT and SA achieved better removal for two compounds, namely salicylic acid (80%) and pentachlorophenol (90%). Both VA and SA achieved above 95% degradation of enterolactone, which compares favorably with 45–70% degradation achieved in absence of mediators (**Figure 5.3**).

Of the 17 non-phenolic compounds, degradation of four compounds *viz* metronidazole, benzophenone, amitriptyline and octocrylene, was at least 90%, regardless of the mediator type (**Figure 5.4**). For the remaining compounds, VA (at 0.5 mM) achieved better degradation for 10 compounds compared to SA and HBT. SA (at 0.5 mM) performed the best for the degradation of two compounds, namely naproxen and primidone. It is well-known that the herbicide atrazine is resistant to laccase catalyzed degradation [22]. Compared to other redox-mediators, HBT was particularly efficient (>99%) for the degradation of atrazine. Although a superior ability of VA compared to other mediators for the degradation of non-phenolic TrOCs has been reported previously in a batch enzymatic bioreactor spiked with four TrOCs [27], the

effectiveness of VA for the degradation of a broad spectrum of non-phenolic TrOCs is demonstrated for the first time in this chapter.

5.4.4.1.1. Impact of redox-mediator concentration

Redox-mediator dose can affect TrOC degradation by changing the abundance, stability and reversibility of the generated radicals [40]. Therefore, the impact of two mediator concentrations (0.25 and 0.5 mM) on ORP, TrOC degradation, and enzyme stability was investigated during preliminary screening of redox-mediators.

Concentration-dependent improvement in the degradation of 18 TrOCs (5 phenolic and 13 non-phenolic compounds, **Figure 5.5**) was observed in MD-EMBR. The highest improvement in the degradation of TrOCs was achieved at 0.5 mM. Notably, increasing the concentration of SA, HBT and VA from 0.25 to 0.5 mM improved TOC degradation by up to 7, 15 and 25%, respectively (**Figure 5.5**). This corresponds well with the respective increase of 2, 5 and 15% of the reaction media ORP (**Figure 5.6**). On the other hand, degradation of 8 phenolic and 4 non-phenolic TrOCs in MD-EMBR was comparable at all the tested mediator concentrations (**Appendix Figure 5-1**). For instance, HBT achieved over 99% degradation of atrazine in MD-EMBR irrespective of the mediator concentration. This is consistent with HBT performance reported in case of UF-EMBR [22].

In general, the degradation of TrOCs that are easily amenable to laccase (**Appendix Figure 5-1**) does not improve significantly (less than 5% in this chapter), while the degradation of resistant TrOCs may improve with the increase in mediator concentration and may reach a plateau beyond a certain mediator concentration. However, the mediator concentration beyond which no improvement occurs may depend on the type of mediators as well as the target TrOCs [38, 41].

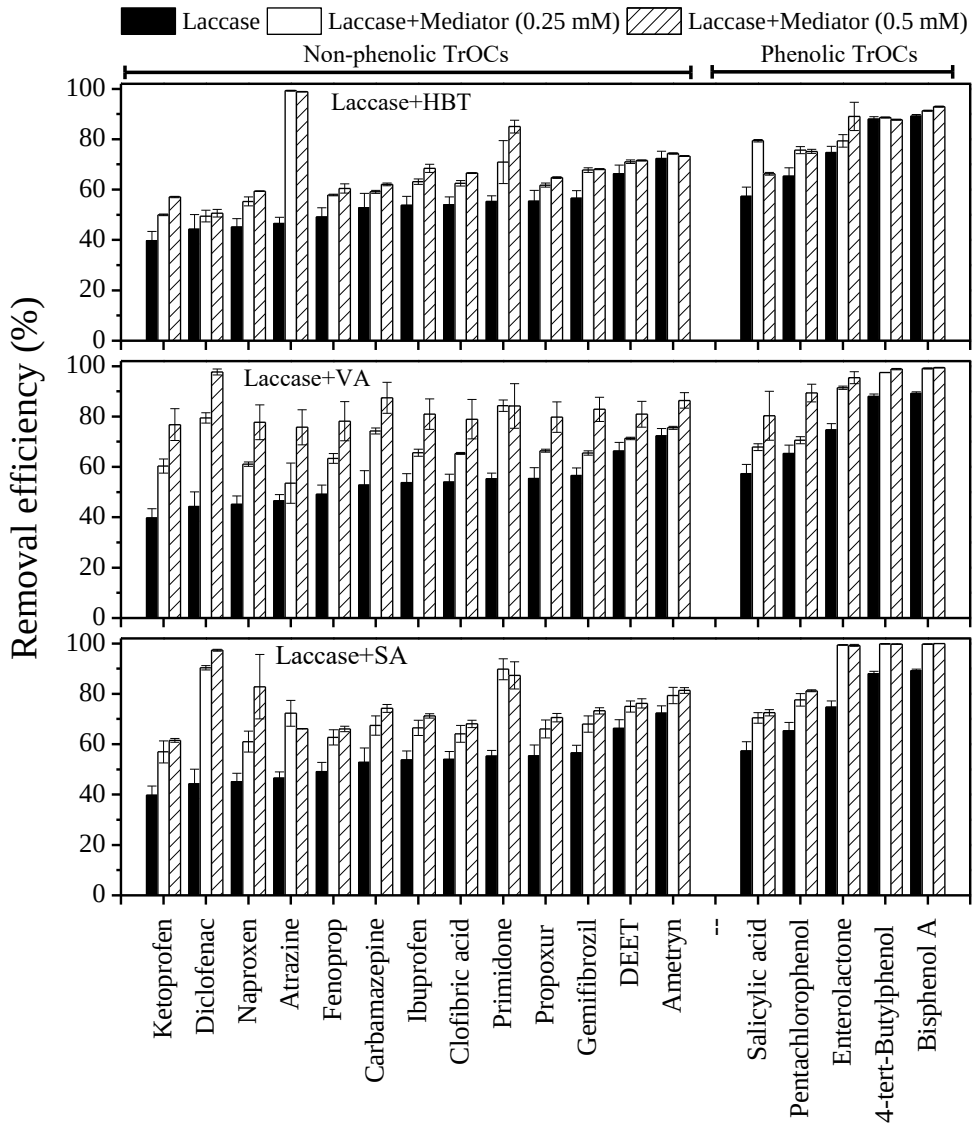

Figure 5.5. Impact of redox-mediator concentration (0.25 and 0.5 mM) on the degradation of TrOCs in the MD-EMBR operated for 12 h. Error bars indicate the standard deviation of duplicate samples. Operating conditions of the MD-EMBR are given in the caption of **Figure 5.1**. Only those TrOCs showing mediator concentration-dependent improvement in their degradation are shown here. For remaining TrOCs, results are given in **Appendix Figure 5-1**.

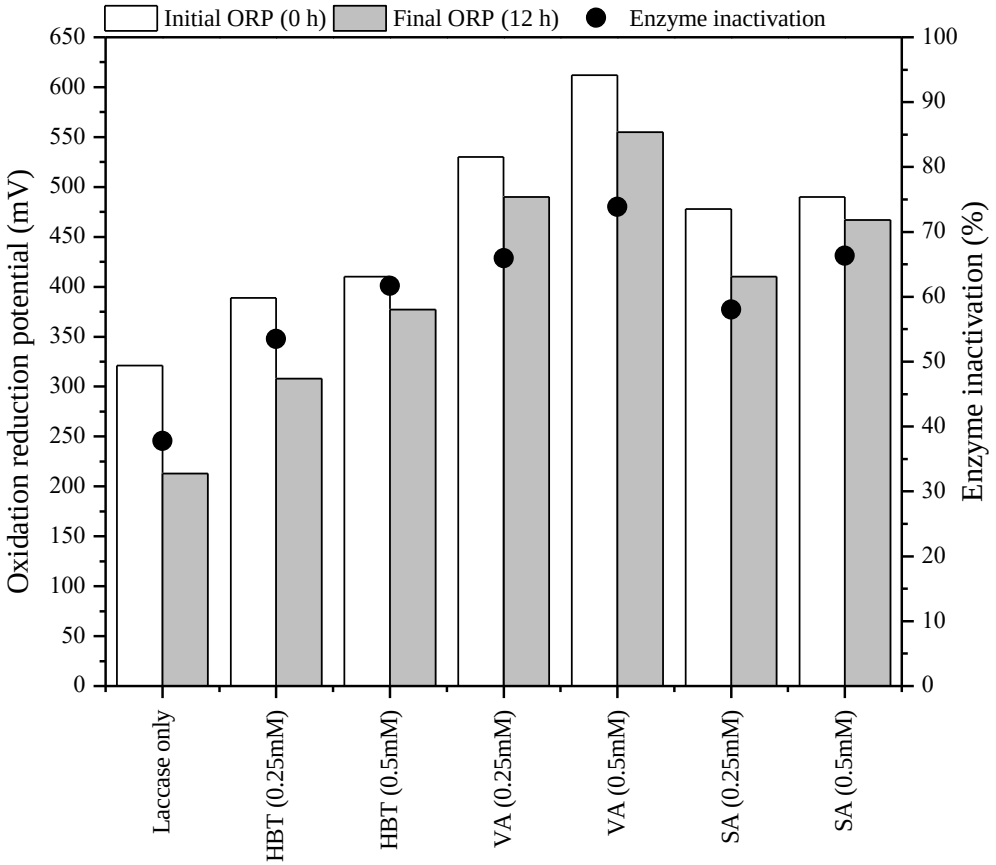

Figure 5.6. Effect of mediator type and concentration on oxidation reduction potential (ORP) and laccase inactivation in the MD-EMBR operated for 12 h. Operating conditions of the MD-EMBR are given in the caption of **Figure 5.1**.

5.4.4.1.2. Effect of mediators on enzyme stability

In this experiment, a gradual inactivation of laccase was observed despite the absence of any known chemical inhibitors in the synthetic wastewater (**Figure 5.6**). In the absence of redox-mediators, a 37% laccase inactivation was observed over a period of 12 h. This was possibly due to the blockage of the active enzyme sites by the charged metabolites and/or hydraulic stress during membrane filtration [38, 42]. Since the MD membrane can conceptually retain all non-volatile organics including the transformation products/radicals, laccase inactivation with or without the presence of redox-mediators can be expected. The extent of laccase inactivation increased further when the mediators were added (61, 66 and 73 for HBT, SA and VA, respectively, each at a concentration of 0.5 mM). The highly reactive radicals generated from mediators can enhance the degradation of TrOCs but at the same time may inactivate laccase [43]. Purich [21] suggested that the metabolites from the oxidation of substrate and/or mediators could react with enzyme to form non-productive complexes, thereby inactivating the enzyme.

The extent of laccase inactivation also depends on the concentration of redox-mediators. For instance, Khlifi-Slama et al. [43] observed a gradual increase in the inactivation of laccase from

Trametes trogii following a stepwise increase in the concentration of HBT from 0.1–10 mM. In another study, increasing SA concentration from 0.1–1 mM resulted in aggravated inactivation of laccase from *Trametes versicolor* [39]. These results suggest that the degree of laccase inactivation is strongly influenced by redox-mediator concentration. Indeed, loss in laccase activity was increased by 7, 9 and 11% in MD-EMBR due to the increase in the concentration of HBT, SA and VA, respectively, from 0.25 to 0.5 mM (**Figure 5.6**). Although laccase activity was greatly affected in the presence of redox-mediators, it was compensated by the improvement in TrOC degradation (**Figure 5.5**). For example, the highest drop in laccase activity was observed in the presence of VA (**Figure 5.6**), but it outperformed SA and HBT in terms of enhanced TrOC degradation (**Figure 5.5**).

5.4.4.2. TrOC degradation following VA and SA addition during long-term operation

During long-term operation of MD-EMBR (**Section 5.4.3**), efficient degradation (95-99%) by MD-EMBR was observed for 13 out of the 30 TrOCs, while the remaining TrOCs were moderately removed (44-75%). While these removal rates compare favorably with that in previous reports, two naturally occurring redox-mediators, namely SA and VA, were selected based on their performance during preliminary screening (**Section 5.4.4.1**). SA and VA were added to the EMBR separately and as a mixture in an attempt to further improve removal of the recalcitrant TrOCs.

Oxidation of VA and SA by laccase produces highly reactive aminoxyl and phenoxyl radicals, respectively, that have higher ORP than laccase. Moreover, these radicals act as an electron shuttle between the substrate and laccase, thereby improving the degradation of the substrate *i.e.*, target pollutants [27]. In a study by Weng et al. [44], addition of SA increased the ORP of the enzyme solution, consequently improving the degradation of sulphonamide antibiotics. Similarly, an increase in ORP was accompanied by an improved degradation of atrazine, pentachlorophenol, naproxen and oxybenzone following the addition of VA at a concentration of 0.5-1 mM in a batch enzymatic bioreactor [27]. In the current experiment, the ORP of EMBR-media increased from 0.3 to 0.39 and 0.45 V following the addition of SA and VA, respectively. This was accompanied by significant improvement in TrOC removal: an increase of 5-54% depending on the molecular structure of TrOCs and redox-mediator type as discussed below (**Figure 5.7**).

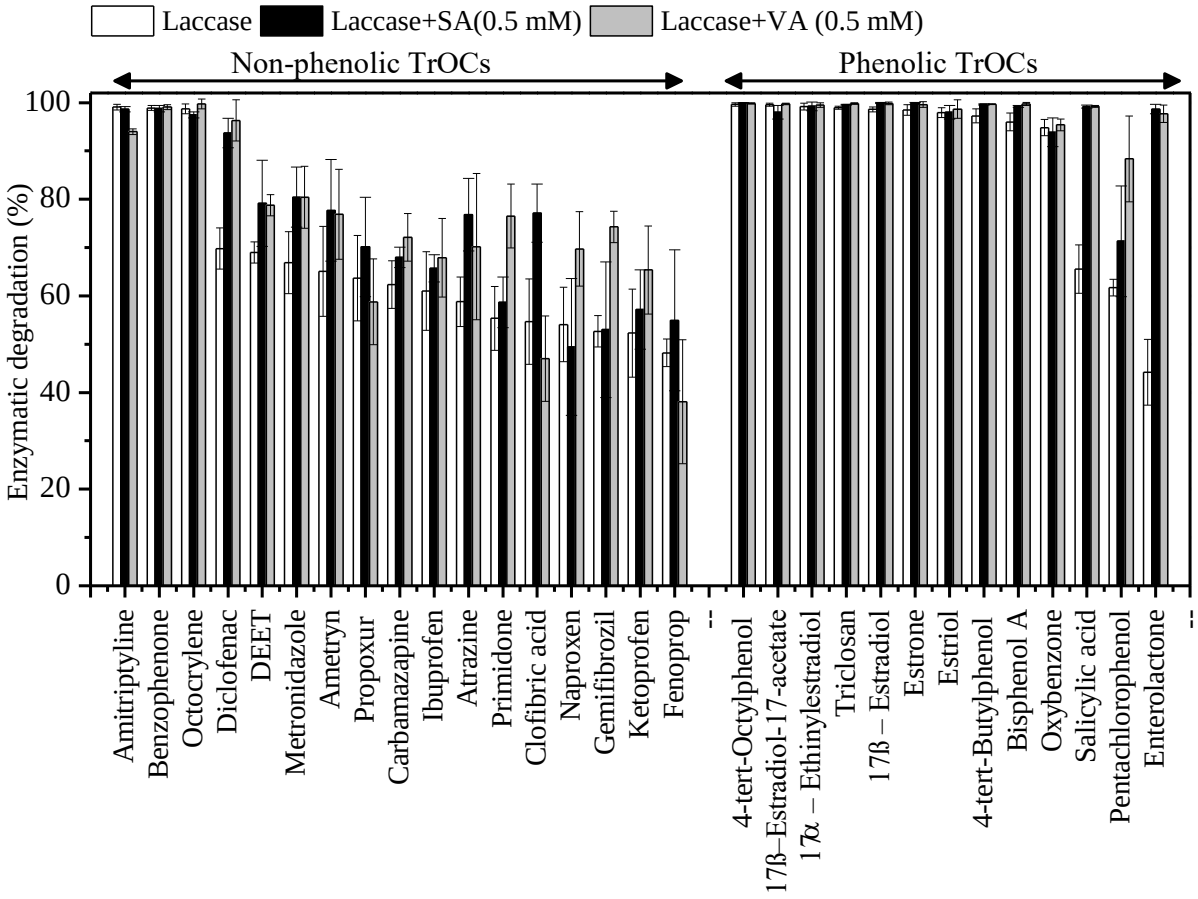

Figure 5.7. Enzymatic degradation of 30 TrOCs following the addition of two redox-mediators viz SA and VA separately at 0.5 mM in MD-EMBR operated for a period of 60 h (i.e., 2×HRT). SA or VA was introduced only at the start of MD-EMBR operation. Data presented as average±standard deviation (n=4). Operating conditions of the MD-EMBR are given in the caption of **Figure 5.1**.

The performance of different mediators for enhanced TrOC degradation has been reported in only a few batch or UF-EMBR studies [26, 27, 38]. In previous studies, a continuous supply of redox-mediator was required to sustain UF-EMBR operation, because UF membranes cannot retain redox-mediators [26, 39]. A uniqueness of this chapter is that it demonstrates the effect of the single dose of mediators on TrOC degradation following the complete retention of laccase, TrOCs and mediators by the MD membrane. SA and VA demonstrated substrate specific improvements in the degradation of TrOC that were moderately degraded by laccase-only (**Figure 5.3**). Of the 17 moderately degraded TrOCs (**Figure 5.3**), the laccase-VA system achieved better degradation for six compounds namely, ketoprofen, gemfibrozil, naproxen, primidone, carbamazepine and pentachlorophenol. By contrast, the laccase-SA system performed best for four compounds*;* fenoprop, clofibric acid, propoxur and atrazine (**Figure 5.7**). Similar degradation efficiency was achieved by both SA and VA for the remaining TrOCs. A comparison of TrOC fate in laccase and laccase-mediator based MD-EMBR revealed that the molecular structures of TrOCs significantly influence the effectiveness of laccase-mediator systems (**Figure 5.8**).

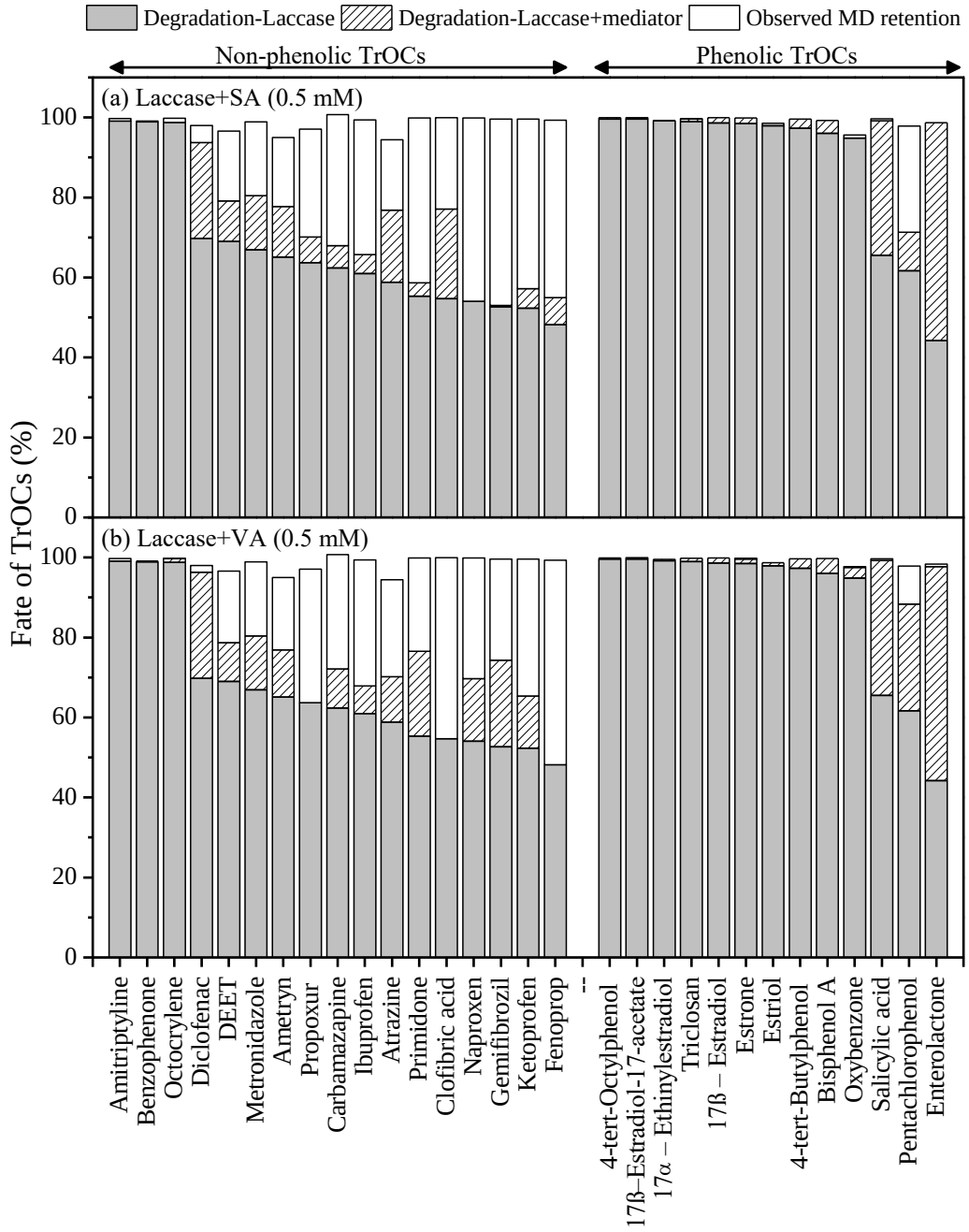

Figure 5.8. Fate of TrOCs during MD-EMBR operation with and without the addition of redox-mediators. Operating conditions of the MD-EMBR are given in the caption of **Figure 5.1**.

5.4.4.3. Effect of mediator mixture on TrOC degradation

Since in this experiment, SA and VA showed different patterns of TrOC-specific degradation-improvement during long-term operation of MD-EMBR (**Figure 5.7**), it was envisaged that a mediator-mixture would have further beneficial effects. Degradation of the phenolic TrOCs, which were already well removed by laccase-only, remained unaffected when a SA-VA mixture was used. The whole set of data is provided in **Appendix Figure 5-2**. However, compared to either SA-laccase or VA-laccase, the SA-VA-laccase system did not improve the

degradation of any TrOCs (**Figure 5.9**). Furthermore, in comparison to TrOC degradation by laccase-only, the SA-VA-laccase system achieved somewhat reduced degradation of six pharmaceuticals, namely ketoprofen, naproxen, clofibric acid, primidone, carbamazepine and ibuprofen (**Figure 5.9**).

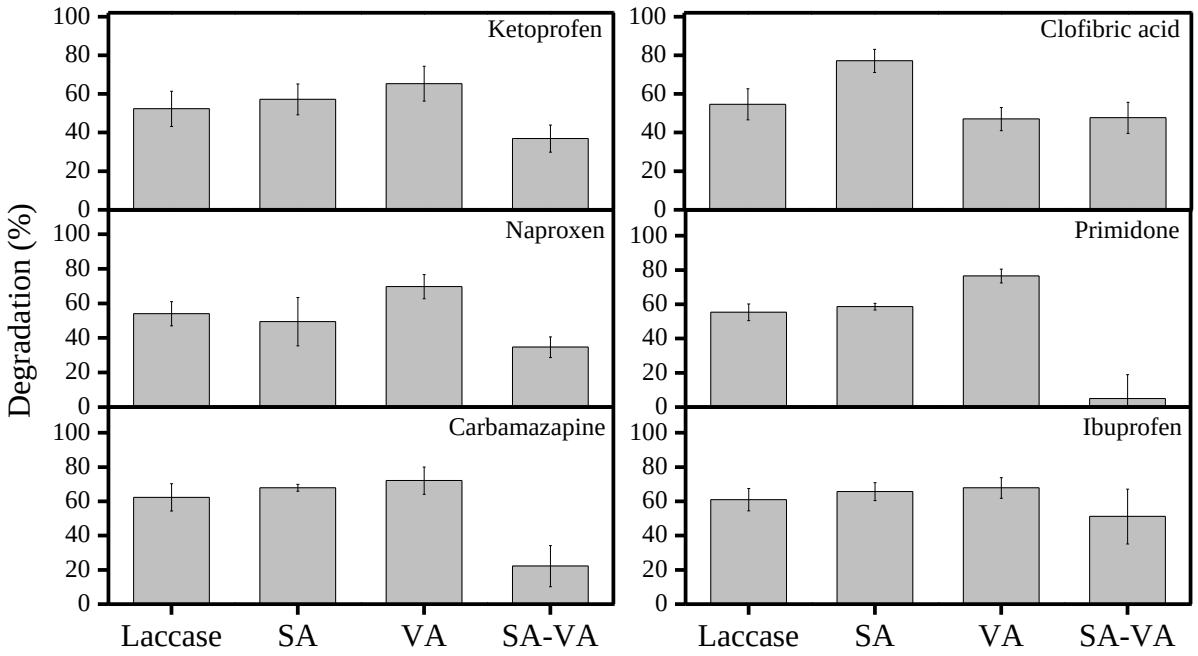

Figure 5.9. Effect of individual mediators and their mixture on the degradation of selected non-phenolic TrOCs, showing reduced performance when mediator mixture was used. Data presented as average±standard deviation (n=4). Effect of mediator mixture (i.e., SA and VA) on all the tested TrOCs (*i.e.*, phenolic and non-phenolic) is shown in **Appendix Figure 5-2**.

The performance of mediator mixtures has rarely been studied for the removal of TrOCs. Previously, Jeon et al. [45] observed in batch tests that vanillin and acetovanillone mixture did not improve the degradation of pentachlorophenol, while enhanced pentachlorophenol degradation was found by adding a mixture of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and vanillin or acetovanillone [45]. It is possible that, in mixtures, some mediators can chemically interact with each other instead of acting as an electron shuttle for laccase. Moreover, simultaneous addition of some mediators can reversibly inhibit laccase, thereby inhibiting electron transfer between laccase and TrOCs [45, 46]. Indeed, laccase inactivation was significantly increased following the addition of SA-VA mixture (**Figure 5.10**). The current work demonstrates for the first time through continuous operation of the MD-EMBR that although VA and SA outcompete many other mediators tested to date [27], using them together may be counterproductive. Further studies to screen redox-mediators and their mixtures are recommended, but that is beyond the scope of the current experiment.

Laccase activity in enzymatic bioreactors may be affected by various physicochemical and biological factors [21, 29]. Transformation byproducts or charged metabolites formed following the degradation of TrOCs can block the active sites of the laccase. Moreover,

hydraulic stress during MD-EMBR operation can also cause laccase inactivation [38]. Although some laccase inhibition was observed during continuous operation of the MD-EMBR (**Figure 5.10**), a stable operation could be sustained by reinjecting as little as 275-400 µL laccase solution per liter of reactor (working) volume every 12 h.

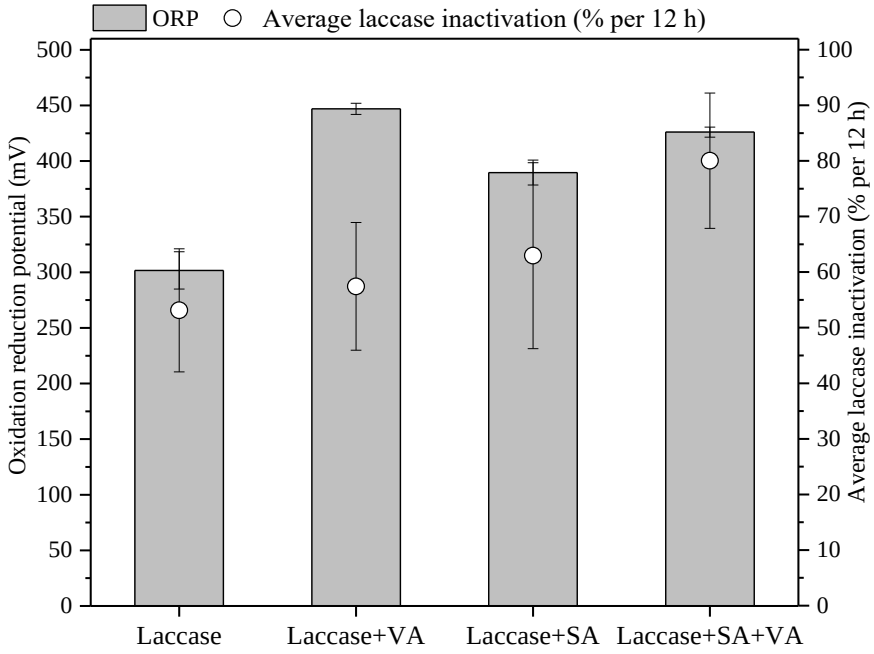

Figure 5.10. Oxidation reduction potential (ORP) and laccase inactivation percentage with and without the addition of redox-mediators. Data presented as average±standard deviation (n=2 for ORP; and n=5 for laccase inactivation). Time course of enzymatic activity during all experiments is given in **Appendix Figure 5-3**.

It may be noted that the MD membrane can theoretically retain all non-volatile compounds including redox-mediator derived highly active radicals along with laccase and TrOCs. The radicals enhance laccase-catalyzed TrOC degradation [35], but can also instigate laccase inactivation. It has been suggested that the highly reactive radicals produced due to the oxidation of mediators can react with laccase, consequently converting them into non-productive complexes [21, 38, 47]. Indeed, the extent of laccase inactivation increased during MD-EMBR operation after mediator addition. This data is available in **Appendix Figure 5-3**. The average laccase inactivation was 53±11% (no. of laccase injections, n=4) during MD-EMBR operation in absence of mediators, while the loss in laccase activity was 57±11, 62±16 and 80±12% (n=4) after the addition of a single dose of VA, SA and SA-VA mixture, respectively (**Figure 5.10**). To date higher laccase inactivation in presence of mediators has mostly been reported in small scale batch enzymatic bioreactors. For instance, Nguyen yet al. [39] reported rapid laccase inactivation following the addition of SA at the tested concentrations of 0.1-1 mM in a batch enzymatic bioreactor. In another study, a complete loss of enzymatic activity was observed in a batch enzymatic bioreactor by adding VA at a concentration of 0.5 mM [27]. In the current experiment, although laccase activity was

significantly affected in the presence of redox-mediators, it was compensated for by the improvement in TrOC degradation (**Figure 5.8**).

5.4.5. Permeate toxicity

Laccase-catalyzed degradation of TrOCs, particularly in the presence of mediators, produces reactive radicals and transformation products that may increase the toxicity of the treated effluent [26, 48]. In this experiment, the overall bacterial toxicity of the media in the enzymatic bioreactor and MD-permeate (*i.e.*, final effluent) was measured at the end of each EMBR run (**Table 5.2**). Of the three mediators tested during preliminary assessment, SA significantly increased the toxicity of the solution in the enzymatic bioreactor, whereas HBT and VA showed no effect on toxicity levels (**Table 5.2**). Compared to the background toxicity of the mixture of laccase and TrOCs in the enzymatic bioreactor of MD-EMBR (<1 to 1.8 rTU; n = 2), toxicity in the enzymatic bioreactor due to addition of HBT, VA and SA ranged from <1 to 1.7 rTU (n = 2), 3.3 to 3.9 rTU (n = 2) and 109 to 116 rTU (n = 2), respectively.

Table 5.2. Toxicity of the bioreactor mixture and permeate following treatment of TrOCs with different mediators in MD-EMBR, expressed as relative toxic unit (rTU). The limit of detection of the toxicity assay was 10% inhibition of luminescence (*i.e.*, 1 rTU). 'NA': not available

Reaction media	**Toxicity in enzymatic bioreactor (rTU)**		**Toxicity of the permeate (rTU)**
	12 h	60 h	
TrOCs + Laccase	<1 – 1.8	4.4 – 5.0	<1
TrOCs + Laccase + HBT (0.5 mM)	<1 – 1.7	–	
TrOCs + Laccase + VA (0.5 mM)	3.3 – 3.9	12.8 – 15	<1
TrOCs + Laccase + SA (0.5 mM)	109 – 116	61.4 – 66.3	<1
TrOCs + Laccase + SA (0.25 mM) + VA (0.25 mM)	NA	119.4 – 136	<1

At the conclusion of long-term operation of MD-EMBR, the media in the enzymatic bioreactor showed an overall toxicity of 4.5-5, 12.8-15, 61.4-66.3, and 119.4-136 rTU (n=2) in presence of laccase, laccase-VA, laccase-SA and laccase-SA-VA, respectively. The observed increase in toxicity due to addition of VA and SA are consistent with previous studies [26, 27], however, the toxicity in relation to mediator mixtures is reported for the first time in this chapter. A significantly increased toxicity following the addition of SA-VA mixture was observed. Despite the increase of toxicity in the enzymatic bioreactor, MD-EMBR permeate toxicity was below the limit of detection (*i.e.*, rTU <1) during all experiments, evidencing that in addition to laccase and TrOCs, the MD system retained reactive radicals and transformation products, which cause bacterial toxicity. This is an added advantage of integrating a high retention membrane with an enzymatic bioreactor.

5.4.6. Permeate flux of MD-EMBRs

The driving force of permeate flux in MD is the difference between feed and distillate temperature. Ideally, feed and distillate temperature is maintained at over 50 and 20–25 °C, respectively to obtain a permeate flux of approximately 10 L/m^2 h [1, 3]. In this experiment, however, to avoid thermal inhibition of laccase [49], temperature of the enzymatic reactor and permeate tank was kept at 30 and 10 °C, respectively. A stable permeate flux of around 4 L/m^2 h was observed during all experiments (**Appendix Figure 5-4 and 5-5**), suggesting that membrane fouling did not occur during the operation period. This level of flux is consistent with the feed temperature employed. Notably, during short-term operation (12 h), the average permeate flux for laccase only, laccase-HBT, laccase-VA and laccase-SA was 3.69 ± 0.44 L/m^2 h, 3.89 ± 0.63 L/m^2 h, 3.92 ± 0.62 L/m^2 h and 3.86 ± 0.66 L/m^2 h, respectively, confirming negligible impact of different type of mediator addition on membrane flux (**Appendix Figure 5-4)**. In this experiment, the mass transfer coefficient (K_m) of the DCMD, which was calculated based on the method described by Nghiem et al. [50], ranged from 1.22 to 1.28 ($\times 10^{-3}$) L/m^2 h Pa. This value is in good agreement with that in previous studies [3, 51]. Thus, this chapter shows both stable membrane hydraulic performance and improved enzymatic degradation of TrOCs following their complete retention by the MD membrane.

During prolonged continuous operation, the performance of the MD process can be affected by the loss of hydrophobicity of the MD membrane [1, 3]. Therefore, the integrity of the MD membrane was assessed by measuring the contact angle of the membrane after each experiment. The contact angle *i.e.*, the hydrophobicity was found to be not significantly affected (**Figure 5.11**), confirming the suitability of combining the MD membrane with the EMBR.

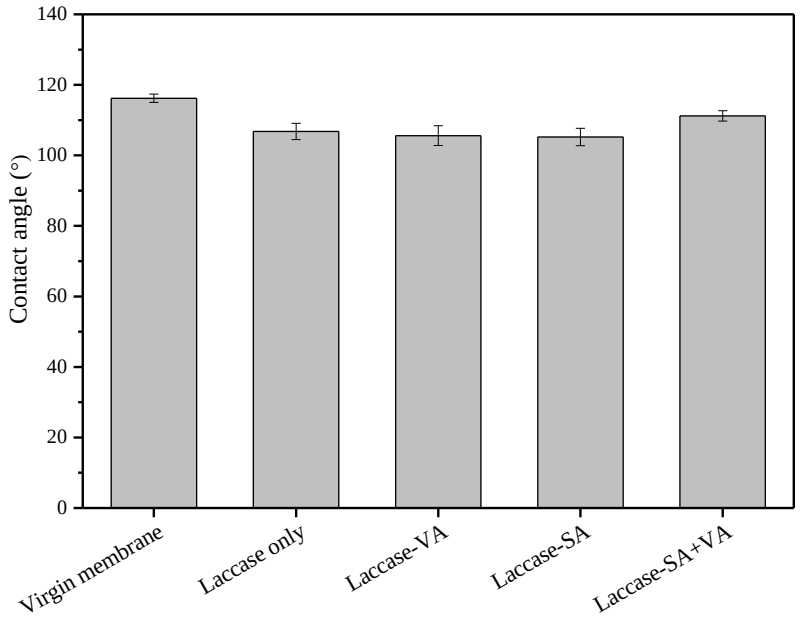

Figure 5.11. Contact angle of the membrane before and after using it for EMBR operation of 60 h. Error bars represent the standard deviation of three repeated measurements.

5.5. Conclusion

Laccase-catalyzed degradation of a broad spectrum of trace organic contaminants (TrOCs) by a membrane distillation (MD) – enzymatic membrane bioreactor (EMBR) was investigated. Initially, the preliminary performance of MD-EMBR was assessed in a series of 12 h experiment. Based on permeate quality, MD-EMBR achieved 90–99% TrOC retention. Degradation of TrOCs varied (40–99%) depending on their molecular properties such as electron withdrawing functional groups (EWGs), and electron donating functional groups (EDGs). High degradation (above 90%) of TrOCs containing EDGs in their chemical structure was observed in the MD-EMBR, while those containing EWGs in their molecular structure were moderately degraded (40–75%). During preliminary assessment, performance of three redox-mediators, namely syringaldehyde (SA), violuric acid (VA) and 1-hydroxybenzotriazole (HBT) was also screened. The results suggest that VA at 0.5 mM concentration was the most effective redox-mediator for improving the degradation of phenolic and non-phenolic TrOCs. In addition, it was observed that the degradation of non-phenolic compounds in laccase-mediator system was strongly influenced by the tested concentration of the redox-mediators. TrOC degradation in the MD-EMBR during long-term operation was also studied for assessing the process stability. The MD component effectively retained TrOCs (94-99%) in the EMBR during long-term operation, facilitating their continuous biocatalytic degradation. The comparison of TrOC degradation by the MD-EMBR after 12 h and 60 h suggests that TrOC degradation was stable. The addition of two redox-mediators, namely SA and VA, further improved TrOC degradation. However, a mixture of redox-mediators showed a reduced

performance for a few pharmaceuticals such as primidone, carbamazepine and ibuprofen. This observation disapproved the hypothesis - mixture of different redox-mediators may achieve better improvement in TrOC degradation than a single redox-mediator. Redox-mediator addition increased the toxicity of the media in the enzymatic bioreactor, but the membrane permeate (*i.e.*, final effluent) was non-toxic, suggesting an added advantage of coupling MD with EMBR. Hydraulic performance of MD-EMBR was stable during all experiments, and membrane wetting, or fouling was not observed.

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Chapter 6: Laccase – persulfate assisted degradation of TrOC by nanofiltration – enzymatic membrane bioreactor (NF-EMBR)

This chapter is based on the following publication:

Asif, M.B., Van De Merwe, J.P., Leusch, F.D., Pramanik, B.K., Price, W.E., Hai, F.I. 2019. Elucidating the performance of an integrated laccase- and persulfate-assisted process for degradation of trace organic contaminants (TrOCs). Submitted to *Environmental Science: Water Research & Technology*

6.1. Introduction

Laccases (EC 1.10.3.2) are copper containing oxidoreductase enzymes and can effectively catalyse the oxidation or degradation of a wide range of aromatic pollutants such as phenols by using molecular oxygen as a co-factor [1-3]. The active sites of laccase contain four copper ions as per following distribution: (i) one copper ion at the Type I active site; (ii) one copper ion at the Type II active site; and (iii) two copper ions at the Type III active site. The degradation of a substrate occurs at the TI active site that acts as the primary electron acceptor. The electron accepted by the Type I active site is transferred to the Type II and Type III active sites, where molecular oxygen is reduced to water [4-6].

In the last decade, laccase-catalysed degradation of trace organic contaminants (TrOCs) such as pharmaceuticals, ingredients of personal care products and industrial chemicals has gained considerable attention [7, 8], because the occurrence of TrOCs in aquatic ecosystems could be potentially harmful to aquatic life and human health [9, 10]. Notably, laccase-catalysed degradation has been reported to be effective for a broad variety of TrOCs as compared to conventional biological processes [11, 12]. Recent studies have also demonstrated that the performance of the laccase-catalysed treatment system is mainly governed by the physicochemical properties of target TrOCs such as chemical structure and hydrophobicity [7, 13]. In general, TrOCs containing a phenolic moiety or electron donating functional groups (EDGs) are effectively degraded (70-99%) by laccase, while degradation of TrOCs containing electron withdrawing functional groups has been reported to be unstable/poor [7, 11].

The application of laccase in continuous systems such as wastewater treatment plants remains a challenge, since laccase is easily washed out with the treated effluent. This loss of laccase in a continuous treatment system could be effectively controlled by integrating a membrane of appropriate pore size with enzymatic bioreactor. Indeed, Lloret et al. [14] and Nguyen et al. [15] developed an enzymatic membrane bioreactor (EMBR) by coupling an ultrafiltration (UF) membrane with an enzymatic bioreactor, and reported effective retention (>99%) of laccase by the ultrafiltration membrane. Interestingly, Nguyen et al. [13] observed the formation of an enzyme gel-layer formed on membrane surface following the continuous permeation of bioreactor media, which adsorbed a few hydrophobic TrOCs (log D>3) such as oxybenzone and improved their degradation by laccase in EMBR. They attributed enhanced degradation to the simultaneous adsorption of TrOCs and laccase on membrane surface, thus promoting the interaction of adsorbed pollutants with laccase active sites [13]. This observation led to the development of high retention (HR) nanofiltration (NF) - and membrane distillation (MD)-EMBRs [5, 16]. During the operation of HR-EMBR, laccase and TrOCs are simultaneously retained by the high retention membrane separation process, thereby providing a prolonged contact time between laccase and TrOCs for enhanced degradation. In a recent study, Asif et al. [5] compared the performance of UF- and NF-EMBR for the treatment of synthetic wastewater containing a mixture of TrOCs including sulfamethoxazole, carbamazepine, diclofenac, atrazine, and oxybenzone under identical operating conditions. As compared to UF-EMBR, they achieved 15–30% better degradation of the selected TrOCs in NF-EMBR [5]. Despite the effective removal (92-99%) of TrOCs achieved by NF-EMBR based on the

permeate quality, degradation of TrOCs (except for oxybenzone) by laccase in the bioreactor ranged between 30 and 80% [5]. The partially degraded TrOCs accumulate in the bioreactor of NF-EMBR over time, and could increase the toxicity of NF-concentrate, consequently making the process of NF-concentrate disposal complex. Therefore, considerable efforts are required to further improve the extent of TrOC degradation within the bioreactor of HR-EMBR.

Degradation of TrOCs in enzymatic treatment systems could be improved by introducing a redox-mediator that is a low molecular weight phenolic compound and can act as electron carrier between laccase and target compounds [4]. In a study by Ashe et al. [17], efficacy of seven redox-mediators such as 1-hydrozybenzotriazole (HBT), violuric acid (VA), syringaldehyde (SA) and 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) for improving TrOC degradation was assessed in laccase-catalysed treatment system. They observed that effectiveness of redox-mediator strongly correlated with its type and concentration. For instance, ABTS achieved the highest degradation for phenolic TrOCs, while HBT and VA were the best redox-mediators for non-phenolic TrOCs [17]. In another study, performance of SA and HBT was compared and elucidated in a continuous-flow UF-EMBR. The tested redox-mediators were found to achieve TrOC-specific improvement in their degradation [13]. However, the feasibility of laccase-mediator based treatment systems is severely affected due to significant laccase inactivation, elevated toxicity of treated effluent and high replenishment costs [4].

Instead of redox-mediators, an alternative and innovative approach could be to develop an integrated treatment system by combining an advanced oxidation process (AOPs) with laccase-catalysed degradation process in a bioreactor. AOPs can be either hydroxyl radical-based such as ultraviolet (UV) photolysis [18], or sulphate radical-based such as activated persulfate (PS) assisted-oxidation process [19, 20]. In recent years, sulphate radical-based AOPs has gained significant attention due to its effectiveness for a wide range of TrOCs such as pharmaceuticals and personal care products in various conditions [20]. To-date, a combined laccase/AOP assisted treatment system has yet to be developed and studied.

Persulfate (PS) is stable at room temperature (*i.e.*, 20 ^{o}C), and requires an activator such as heat (as low as 30 ^{o}C), transition metals (*e.g.*, iron) and UV light to generate highly reactive $SO_4^{-\bullet}$ radicals. Depending on the type of activator, PS produces one (*e.g.*, in presence of transition metals, *see* Equation 1) or two (*e.g.*, in presence of heat or UV light, *see* Equation 2) $SO_4^{-\bullet}$ radicals [20, 21]. It is worth mentioning that phenolic compounds [22], humic substances containing quinone functional groups [23], graphene [24], activated carbon [20], ultrasonication [20] and sub-surface minerals [25] have also been reported to activate PS. However, a thorough literature survey suggests that PS activation by using enzymes has not been assessed.

$$S_2O_8{}^{2-} + heat\ or\ UV\ light \rightarrow 2\,SO_4{}^{2-\cdot} \quad (1)$$

$$S_2O_8{}^{2-} + Fe^{+2} \rightarrow Fe^{+3} + SO_4{}^{2-\cdot} \quad (2)$$

PS activation, formation of reactive radicals, and identification of degradation products or metabolites has been predominantly studied, to-date. In addition, performance of PS has been reported for the degradation of a single TrOC at concentration significantly higher than its environmentally relevant concentration [20, 26]. The logical step forward should be to assess the performance of PS for a mixture of TrOCs, because the extent of degradation for a single TrOC may change in a reaction media containing a mixture of TrOCs. Importantly, toxicity and estrogenicity of the treated effluent should be analysed for safe disposal and reuse of the treated effluent.

This chapter elucidates the degradation of a mixture of five TrOCs in a laccase/persulfate (PS) oxidation-assisted nanofiltration membrane bioreactor (NFBR) for the first time. Initially, batch experiments were performed to understand the effect of initial PS concentration and effect of incubation time as well as possible PS activation mechanisms. While discussing the removals achieved by batch and continuous-flow bioreactors, physicochemical properties of TrOCs were also considered to provide an in-depth understanding. Estrogenicity and toxicity of the bioreactor media and membrane permeate were analysed and discussed. Finally, the hydraulic performance of NFBR is presented to confirm the stability of the developed process.

6.2. Hypothesis

- Integrated laccase and PS oxidation processes may synergistically facilitate TrOC degradation
- The extent of degradation in laccase/PS system is governed by TrOC properties and PS concentration
- Enhanced TrOC degradation may result in reduced toxicity and estrogenic activity

6.3. Materials and methods

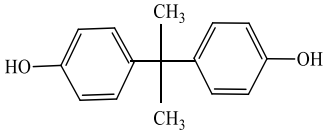
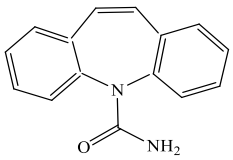
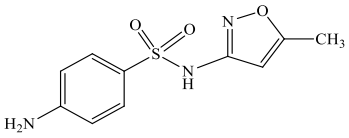
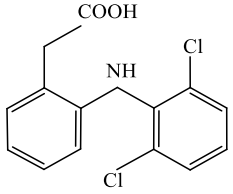
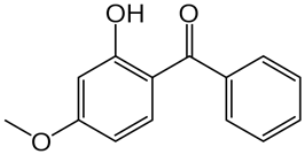
6.3.1. Trace organic contaminants, laccase solution and persulfate

In this experiment, one industrial chemical (bisphanol A) and four pharmaceuticals and personal care products, namely diclofenac, sulfamethoxazole, carbamazepine and oxybenzone were selected based on their ubiquitous presence in wastewater and freshwater bodies [9]. For both batch and continuous experiments, a synthetic wastewater containing the mixture of the selected TrOCs each at a concentration of 500 μg/L in ultrapure Milli-Q water was prepared. All the TrOCs were of analytical grade (purity >98%), and were procured from Sigma–Aldrich (Sydney, NSW, Australia). A stock solution (2 g/L) was also prepared by dissolving the mixture of the selected TrOCs in pure methanol. The TrOC stock solution was kept at –18°C in the dark and used within one month. The main physicochemical properties of the selected TrOCs are presented in **Table 6.1**.

The enzyme solution acquired from Novozymes Australia Pty. Ltd. (Sydney, NSW, Australia) was commercially available laccase from genetically modified *Aspergillus oryzae*. Properties of laccase are presented in **Section 3.3.1 (Chapter 3)**. Reagent grade (purity ≥99%) potassium

persulfate (PS) was purchased from Sigma-Aldrich (Sydney, NSW, Australia). A stock solution (50 mM) of PS was prepared in ultrapure Milli-Q water and stored at 4ºC before use.

Table 6.1. Selected physicochemical properties of the selected TrOCs

TrOCs	Molecular structure	Molecular weight (g/mole)	Water solubility at 25 ºC (mg/L)	Acid dissociation coefficient (pK_a)	log D at pH=7
Bisphenol A		228.29	120	10.29	3.64
Carbamazepine		236.27	220	13.94	1.89
Sulfamethoxazole		253.28	410	5.6	–0.22
Diclofenac		296.15	30	4.18	1.77
Oxybenzone		228.24	100	7.56	3.99

6.3.2. Performance of laccase and persulfate in batch bioreactor

The performance of laccase/PS was initially assessed in batch bioreactor at different PS concentration (*i.e.,* 1, 2, 5 and 10 mM) for an incubation period of 24 h. All the experiments were conducted in 250 mL conical flasks. The concentrated laccase stock solution (70 µL) was diluted to a final volume of 100 mL in conical flasks for maintaining an initial enzymatic activity of 90-95 $\mu M_{(DMP)}$/min. PS and the selected TrOC were added in conical flasks at an initial concentration of 1-10 mM and 500 µg/L, respectively. Actual initial measured TrOC concentrations of bisphenol A, diclofenac, sulfamethoxazole, carbamazepine and oxybenzone were 510±15, 485±10, 525±20, 510±10 and 480±5 µg/L (n=12), respectively. The initial pH of the reaction media was not adjusted during all experiments and was approximately 7. All the conical flasks were incubated in an orbital shaker incubator (Model 8500, Bioline Global Pty Ltd. Australia) at 80 rpm and 25ºC. Triplicate samples were collected at 2, 4, 8 and 24 h for TrOC analysis. Samples for measuring the laccase activity, PS consumption, estrogenic activity and ecotoxicity were also collected at the end of each batch experiment. To verify the contribution of laccase and PS in TrOC degradation, 'control' batch tests were performed in

parallel by studying the performance of laccase alone, PS alone, and PS-heat inactivated laccase.

6.3.3. Continuous nanofiltration-bioreactor setup and experimental protocol

6.3.1.1. Description of experimental setup

For elucidating the performance of accase/PS in continuous-flow mode, a lab-scale cross-flow nanofiltration (NF) setup coupled to a bioreactor (3 L working volume) was used (**Figure 3.1**). A detailed description of the cross-flow NF setup is available in **Section 3.3.2** (**Chapter 3**). A commercially available flat-sheet NF90 membrane (Dow/Filmtec, USA) was used. It was a thin-film composite membrane with polyamide based active layer, and its molecular weight cut-off (MWCO) was 200 Da.

6.3.1.2. Experimental protocol

The experiment was started by compacting the membrane at an initial hydraulic pressure of 10 bar for at least 1 h or until the stabilization of permeate flow rate. The synthetic wastewater (3 L volume) containing the mixture of the selected TrOCs each at a concentration of 500 µg/L was added in the bioreactor of NF filtration setup. Laccase and PS from their respective stock solutions were directly added in the bioreactor to maintain an initial laccase activity of 90-95 $\mu M_{(DMP)}$/min, and PS concentration of 5 mM. The NFBR system was then operated at a hydraulic pressure of 8 bar and cross-flow velocity of 40 cm/s. This resulted in an initial permeate flux of 6.8 L/m^2 h bar. The synthetic wastewater containing TrOC mixture was continuously fed to the bioreactor *via* a peristaltic pump (Masterflex, USA) for a period of 64 h (*i.e.*, 4 × hydraulic retention time, HRT). Triplicate samples from the bioreactor and membrane permeate were collected at 0, 6, 12, 24, 36, 48, 64 h for TrOCs analysis. In addition, samples were obtained regularly every 12 h for measuring the laccase activity and PS consumption in the bioreactor and membrane permeate. At the end of experiment, samples from feed, bioreactor and membrane permeate were collected for the evaluation of estrogenic activity and ecotoxicity as explained in **Section 6.3.4.3.** Hydraulic performance of the NF membrane was studied by monitoring the permeate flux. At the conclusion of the experiment, the membrane was cleaned with Milli-Q water for 1 h to check flux recovery.

Laccase activity has been observed to diminish during continuous operation due to different physicochemical and biological inhibitors as explained previously [5, 19]. A protocol was developed to replenish the laccase activity by adding approximately 150 µL per litre of bioreactor volume every 24 h. Importantly, PS at a concentration of 5 mM was added only once at the start of laccase/PS assisted NFBR operation.

6.3.4. Analytical methods

6.3.4.1. TrOC analysis

TrOC in samples collected from batch and continuous-flow bioreactors was quantified by High-performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan) using a method reported previously [5]. A detail description of this method is available in **Section 3.3.4.1**

(**Chapter 3**). Removal efficiency by laccase/PS ($R_{degradation}$) and the membrane ($R_{degradation+membrane\ retention}$) was measured using equation (3) and (4), respectively:

$$R_{biodegradation} = 100 \times (1 - C_{BR}/C_f) \quad (3)$$

$$R_{(degradation+membrane\ retention)} = 100 \times (1 - C_p/C_f) \quad (4)$$

where, C_f, C_{BR} and C_p are the concentration (µg/L) of a specific TrOC in the feed, bioreactor and membrane permeate, respectively. The mass of TrOCs degraded by laccase/PS during continuous-flow NF-BR operation was calculated as follows:

$$C_f \times V_f = (C_{BR} \times V_{BR}) + (C_p \times V_p) + \text{degradation/transformation} \quad (5)$$

where, V_f, V_{BR} and V_p represents the volume of feed, bioreactor and permeate, respectively.

6.3.4.2. Laccase activity essay and PS concentration measurement

Laccase activity was measured as described in **Section 3.3.4.2** (**Chapter 3**). PS concentration may change following its addition in both batch and continuous-flow bioreactors. The change in PS concentration was measured during each experiment by using a previously developed spectrophotometric method [19, 27].

6.3.4.3. Estrogenic activity and ecotoxicity

Duplicate samples (110 mL each) collected from the batch bioreactors and triplicate samples (110 mL each) collected each from the continuous-flow bioreactor and membrane permeate were extracted using Oasis HLB cartridges and eluted in 5 mL methanol. This resulted in a relative concentration factor of 22 for each sample. Estrogenic activity was analysed by ERα-GeneBLAzer assay (Life Technologies, USA) as described previously [28, 29]. This is an estrogen receptor-mediated reporter gene assay that measures the presence of either estrogens or estrogen mimicking compounds. This assay was carried out in 384-well plate and run in both antagonist and agonist modes. A Fluostar plate reader (BMG Labtech, Germany) was used for measuring the fluorescence at wavelengths of 460 and 520 nm after excitation at 410 nm. The data from the plate reader was presented as the ratio of fluorescence obtained at 460 nm to that obtained at 520 nm. The results were compared with the concentration-effect curve of reference standards and expressed as 17β-estadiol (agonist) and 4-hydroxytamoxifen (antagonist) equivalent concentration. The limits of detection for agonistic activity were 0.35 and 1.4 ng/L for 17β-estradiol (E2-EQ) in batch and continuous flow-experiments, respectively. The detection limit for anti-estrogenicity was 20 µg/L for 4-hydroxytamoxifen (4-OHTMX-EQ) in all experiments. The method for ecotoxicity assay has already been described in **Section 5.3.4.3** (**Chapter 5**)**.**

6.4. Results and discussion

6.4.1. TrOC removal in batch experiments

6.4.1.1. Preliminary performance of integrated laccase/PS system

Laccase is particularly suitable for the degradation of phenolic compounds but can also catalyse the degradation of non-phenolic compounds. The extent of degradation for non-phenolics is dependent on the relative ORP of laccase and target compound [30, 31]. In this experiment, the non-phenolic TrOCs were poorly degraded (less than 15%) by laccase (**Figure 6.1**). At the end of the incubation period of 24 h, laccase achieved 7, 9 and 15% degradation of sulfamethoxazole, carbamazepine and diclofenac, respectively. This recalcitrance of non-phenolic TrOCs could be attributed to their chemical structure. All the tested non-phenolic TrOCs contain strong EWGs. For example, carbamazepine and sulfamethoxazole contain amide ($–NH_2$) functional group, and diclofenac contains both halogen (–X) and carboxylic ($–CH_3$) functional groups in its molecule (*see* **Table 6.1**). These EWGs make TrOCs resistant to laccase because they can release electrons to stabilise the electron deficiency caused by the degradation process [3, 32]. Indeed, poor or unstable removal of non-phenolics during laccase-catalysed degradation is also evident from available literature with reported removal often ranging between 10 and 25% [3, 11, 33].

Although phenols have been recognised as a typical substrate of laccase [7, 32], their effective removal by laccase is not always possible. Out of five tested TrOCs, two compounds (oxybenzone and bisphenol A) contain phenolic moiety in their chemical structures, and their degradation by laccase is TrOC-specific. Laccase achieved 57% and complete degradation for bisphenol A and oxybenzone, respectively (**Figure 6.1**). Almost complete degradation of bisphenol A by laccase in batch enzymatic bioreactors treating the mixture of bisphenol A and diclofenac was reported previously [15]. However, in this experiment, moderate degradation of bisphenol A could be due to the competitiveness among phenolic TrOCs for transferring an electron to active sites of laccase for degradation.

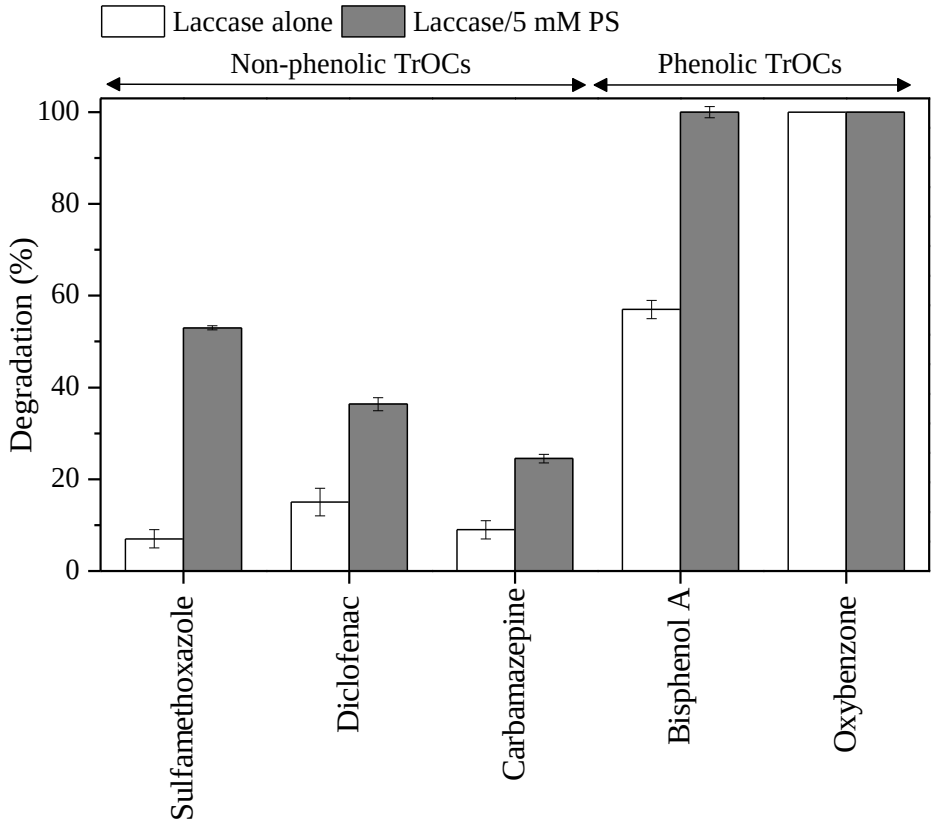


Figure 6.1. Degradation of the selected TrOCs by laccase and laccase/PS in batch tests after an incubation time of 24 h. PS (potassium persulfate) was added at 5 mM concentration, while the initial laccase activity was 90-95 $\mu M_{(DMP)}$/min. Results presented as average ± standard-deviation (n=3). Note: performance of PS alone at 1 mM concentration was assessed in parallel, and no TrOC removal observed.

All TrOCs (except for oxybenzone) were not effectively degraded, thus indicating the need of improvement *via* supplementing the laccase-catalysed degradation process with an oxidizing agent capable of TrOC degradation. In the current experiment, performance of the laccase-catalysed degradation process was assessed by adding 5 mM PS in batch enzymatic bioreactor for an incubation period of 24 h. This significantly improved (15-46%) the extent of TrOC degradation, indicating the complementarity of combining laccase and PS oxidation process. Compared to less than 20% degradation of non-phenolics by laccase, PS achieved 24, 36 and 53% degradation of carbamazepine, diclofenac, and sulfamethoxazole, respectively (**Figure 6.1**). On the other hand, PS addition provided 43% improvement in the degradation of a phenolic plasticizer bisphenol A that was moderately degraded (57%) by laccase. Notably, following treatment with and without PS addition, oxybenzone concentration was below the limit of detection (*i.e.,* 10 µg/L). Performance of an integrated laccase/PS assisted oxidation process for TrOC removal is reported for the first time in this chapter.

It is important to note that an additional agent such as transition metals, heat or UV light is required to activate PS for the generation of $SO_4^{-\bullet}$ and/or $OH^{-\bullet}$ radicals [20]. Despite the absence of any known activators in reaction media, enhanced TrOC degradation by laccase/PS process indicated that PS was activated and produced radicals in the batch laccase/PS system.

For understanding the possible routes of PS activation, mechanism of laccase-catalysed degradation process needs to be revisited. Oxidation of a substrate by laccase occurs following the transfer of an electron from the substrate to the Type I active site of laccase. This is followed by the transfer of electrons to Type II and III active sites, where reduction of the cofactor oxygen to water molecules occurs. During the reduction of O_2, formation of peroxide intermediates has been observed [6]. The production of peroxide intermediates can activate PS to produce reactive radicals [34]. To produce $SO_4^{-\bullet}$ radicals, PS needs an electron from any source [6]. Hence, there is a possibility that both oxygen and PS may have acted as the cofactor and may accept electron from Type II and III active sites of laccase for completing the catalytic cycle. In the current study, the possibility of PS acting as a final electron acceptor was investigated by removing the dissolved oxygen from reaction media *via* autoclaving (**Figure 6.2**). Dissolved oxygen in the reaction media measured using a DO meter (YSI, USA) was less than 0.01 mg/L. In absence of oxygen, TrOC degradation occurred in laccase/PS system but the extent of degradation of all the tested TrOCs (except for oxybenzone) reduced by 5 to 20% as compared to that achieved by laccase/PS system in presence of oxygen. Notably, although TrOC degradation was affected in absence of oxygen, PS activation still happened as evident from the better TrOC degradation by laccase/PS system without oxygen as compared to laccase alone (**Figure 6.2**). An additional batch run was performed by adding heat-inactivated laccase and PS in the bioreactor for a period of 24 h. Like the performance of laccase/PS system, degradation of TrOCs reduced significantly (5-40%) in the heat-inactivated laccase /PS system (**Figure 6.2**). These results indicate that PS activation is possibly caused by the structural components (*e.g.,* a polypeptide chain and carbohydrate moieties) of laccase [35]. This has not been reported in literature, to date. Notably, TrOC degradation was affected more in heat-inactivated laccase/PS system as compared to that achieved by laccase/PS system in absence of oxygen, thus indicating the significance of active laccase in an integrated laccase/PS treatment system. Based on the above observations, PS activation may have caused by both the structural components of laccase as well as the possibility of PS acting as a final acceptor of electrons transferred by Type II and III active sites of laccase.

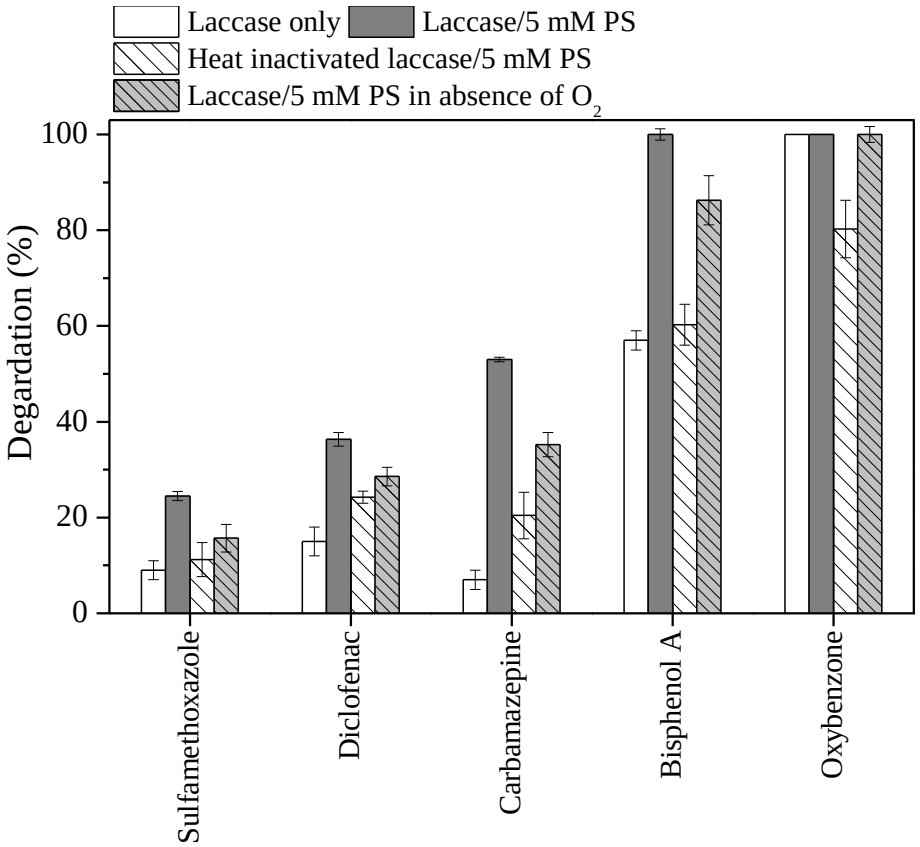


Figure 6.2. Degradation of the selected TrOCs by laccase and laccase/PS in batch tests for understanding the PS activation pathways. PS (potassium persulfate) was added at 5 mM concentration, while the initial laccase activity was 90-95 $\mu M_{(DMP)}$/min. Results presented as average ± standard-deviation (n=3).

6.4.1.2. Effect of PS concentration

The effects of combining laccase and PS are comprehensively demonstrated in the preceding section. For optimization, performance of PS in laccase/PS system was analysed at different PS concentrations of 1-10 mM. In this experiment, degradation of the tested TrOC, except for oxybenzone that was completely degraded at all the tested concentration of PS, improved by increasing the PS concentration from 1 to 10 mM, although the extent of improvement was compound-specific. For instance, degradation of sulfamethoxazole was 23, 36, 53 and 72% at an initial PS concentration of 1, 2, 5 and 10 mM (**Figure 6.3**). Similarly, carbamazepine degradation increased from 11% (at 1 mM PS) to 40% (at 10 mM PS). Since performance of laccase/PS system was studied for the first time, it is not possible to compare the results of this chapter with literature. However, the trend in the improvement of TrOCs with increasing PS concentration seems to be consistent with available literature. According to the available literature, TrOC degradation generally improves with the increase in PS concentration. For instance, Ji et al. [26] reported an improvement of approximately 80% in the degradation of the pesticide atrazine by heat-activated PS after the increase in concentration from 0.1 to 2 mM. In another study, degradation of sulfamethoxazole increased from merely 10 to 70% following the increase in the concentration of bicarbonate-activated PS from 1 to 10 mM [36].

The best PS concentration for achieving the highest degradation was different for the tested TrOCs. It was 1 mM for oxybenzone and 5 mM for bisphenol A, while 10 mM was the most effective PS concentration for diclofenac, carbamazepine and sulfamethoxazole (**Figure 6.3**).

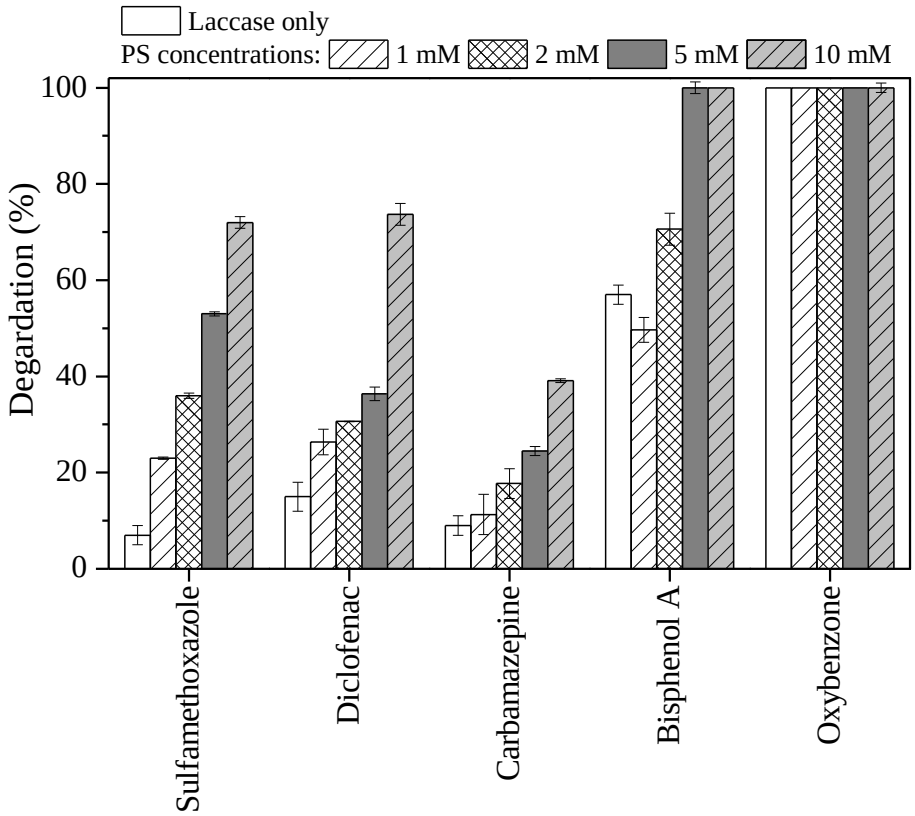


Figure 6.3. Effect of PS concentrations on TrOC degradation in batch laccase/PS system. PS concentration ranged from 1-10 mM, while the initial laccase activity was 90-95 $\mu M_{(DMP)}$/min. Results presented as average ± standard-deviation (n=3).

Laccase inactivation can be a concern during TrOC degradation, requiring intermittent replenishment of laccase. Different physicochemical (*e.g.*, salts and heavy metals) and biological (*e.g.*, organic acids and humic substances) factors can cause laccase inactivation [3, 17]. In absence of any known inhibitor, laccase activity did not reduce significantly (~2%) in this experiment. However, when PS was added in the enzymatic bioreactor, increase in laccase inactivation was observed (**Figure 6.4a**). At the end of the operation of batch laccase/PS system, laccase inactivation was 7% at 1 mM PS concentration, which increased to 16, 18, and 43% following addition of PS at 2, 5 and 10 mM, respectively (**Figure 6.4a**). Laccase inactivation could also be due to the radicals produced by PS that can interact with the active sites of laccase, thereby affecting laccase activity. Previously, redox-mediators were added in enzymatic bioreactor for improving the degradation of TrOCs. Despite the TrOC-specific improvement in degradation, the radicals produced by redox-mediators has been reported to cause rapid laccase inactivation [1, 4, 37]. For instance, laccase was reported to lose 70-80% of its initial activity following the addition of violuric acid and 1-hydrozybenzotriazole separately at 1 mM concentration. Notably, laccase inactivation caused by PS radicals is significantly lower than that reported in presence of redox-mediators.

In addition to laccase inactivation, depletion of PS may occur due to the scavenging reactions in which radicals react with other radicals or nontarget compounds. These scavenging reactions deplete PS by converting sulphate radicals into sulphate ions [38, 39]. In this experiment, PS depletion was insignificant (less than 2%) at a PS concentration of 1-5 mM but increased considerably (36%) in presence of 10 mM PS in laccase/PS system after 24 h of batch experiment (**Figure 6.4b**). Thus, it important to consider the concentration of PS for developing a stable and efficient integrated laccase/PS treatment system.

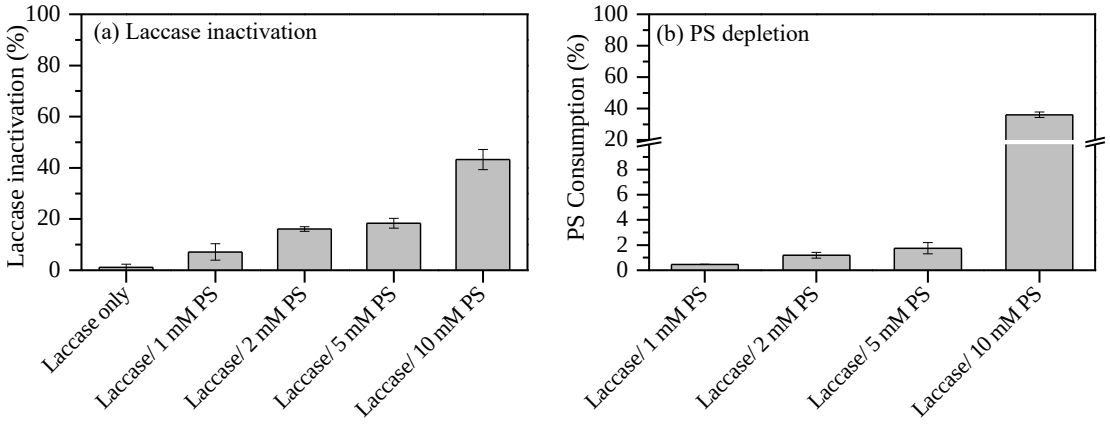


Figure 6.4. Laccase inactivation (a) and depletion of PS (b) at the end of batch tests with and without the addition PS at different concentrations. Error bars represents the standard deviation between duplicate samples.

6.4.1.3. Effect of incubation time

Degradation efficiency of the integrated laccase/PS system was assessed at different incubation periods (*i.e.*, 2, 4 8 and 24 h). To facilitate the discussion, performance of the integrated systems at 2- and 5-mM PS concentration is presented in **Figure 6.5**. Laccase/PS system achieved rapid degradation of both phenolic TrOCs. Complete elimination of oxybenzone was observed after an incubation time of 2 h (at 5 mM PS concentration) and 4 h (at 2 mM Ps concentration). On the other hand, 5 mM PS achieved 100% degradation of bisphenol A after 8 h in laccase/PS system (**Figure 6.5**). Regardless of PS concentration, the maximum degradation for non-phenolics was observed within first 8 h of incubation period, and the degradation slowed down considerably from 8 to 24 h. These results are consistent with the available literature related to the performance of PS for the degradation of an individual TrOC. For instance, when heat-activated PS at 0.5 mM concentration was investigated for the degradation of an antibiotic (penicillin G), its maximum degradation occurred within first 60 min [40]. Similarly, degradation of sulfamethoxazole by PS was not observed to increase significantly after an incubation period of 6 h in a PS/bicarbonate system [36]. The observations related to the incubation time are vital for designing a wastewater treatment system. This is because incubation time is an important parameter to estimate the size of the reactor required for effective treatment, and its overestimation may considerably increase the cost of the treatment system.

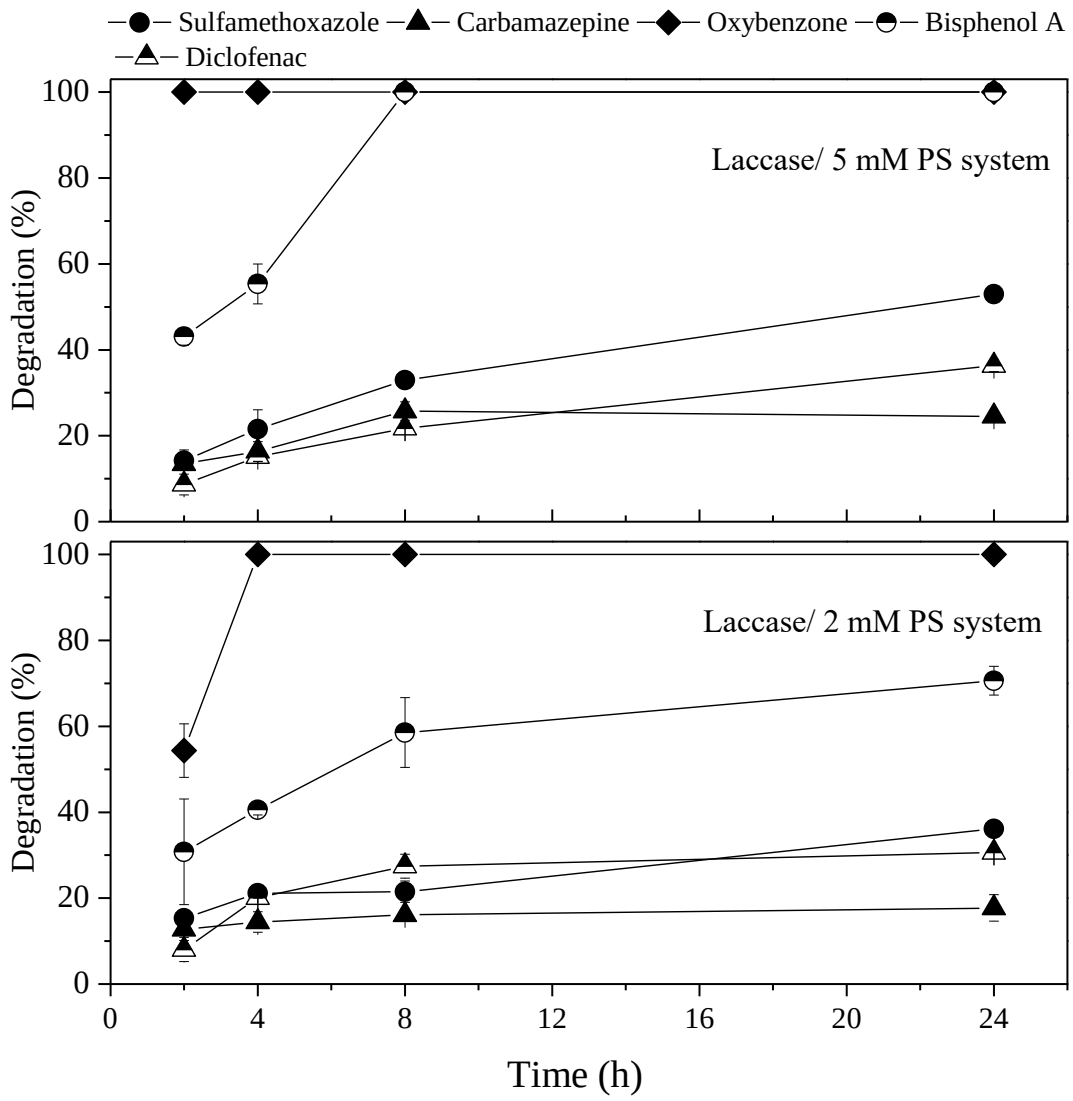


Figure 6.5. Effect of incubation time on TrOC degradation in batch laccase/PS system assessed separately at 2- and 5-mM PS concentration. Initial laccase activity was 90-95 $\mu M_{(DMP)}$/min. Results presented as average ± standard-deviation (n=3).

6.4.1.4. Ecotoxicity and Estrogenic evaluation

Toxicity cannot be estimated by quantifying the actual amount of TrOC in the treated effluent before disposal, because the degradation of TrOCs results in the production of transformation by-products or metabolites that could cause more toxicity than the parent compound. To predict the risk associated with the disposal of treated effluent, bioassays have been developed and reported for quantifying the toxicity [17, 41]. In this experiment, toxicity of the treated effluent was evaluated by measuring the inhibition of luminescence in the naturally bioluminescent bacteria (*Photobacterium leiognathi*) using the BLT-Screen [42]. Consistent with previous studies [13, 41], toxicity following treatment with laccase alone did not increase significantly and ranged between less than 1 and 2.3 rTU (**Table 6.2**). On the other hand, toxicity of reaction media increased from 7.6 to 28.8 rTU in batch laccase/PS systems with the increase in PS concentration (**Table 6.2**). This could be attributed to: the reactive radicals generated by PS; and/or the generation of toxic transformation by-products. Reduction in toxicity following

treatment of a single TrOC by PS has been reported previously [43, 44]. However, toxicity of the media following degradation of one TrOC is not comparable to that obtained from the media containing a mixture of five TrOCs. Indeed, Kortenkamp et al. [45] observed that the toxic effects of the solution containing a mixture of compounds are often higher than the toxic effects of individual compound.

Table 6.2. Estrogenic activity and ecotoxicity of samples collected at the end of different treatment options. Number of samples, n = 2.

Reaction media of different treatment options	Toxicity (rTU)	E2-EQ (ng/L)	4-OHTMX-EQ (µg/L)
TrOCs only	<1	9.7 – 13.9	<20
TrOCs – Laccase	<1 – 2.3	< 1.4	<20
Laccase – TrOCs – Persulfate (1 mM)	7.6 – 11.3	2.3 – 2.6	<20
Laccase – TrOCs – Persulfate (2 mM)	3.9 – 5.6	1.8 – 2.6	<20
Laccase – TrOCs – Persulfate (5 mM)	10.3 – 11.2	3.3 – 3.9	<20
Laccase – TrOCs – Persulfate (10 mM)	24.8 – 28.8	2 – 2.2	<20

Note: The limit of detection of the toxicity assay was 1 rTU
The limit of detection for E2-EQ and 4-OHTMX-EQ was 1.4 ng/L and 20 (µg/L), respectively.
E2-EQ stands for 17β-estradiol equivalent; and 4-OHTMX-EQ stands for 4-hydroxytamoxifen equivalent.

Estrogenic activity was analysed because the prolonged exposure to TrOCs, particularly bisphenol A has been reported to induce endocrine disrupting effects in aquatic life and human [41, 46]. In this experiment, the estrogenic activity before and after treatment was evaluated by using GeneBLAzer ERα-UAS-*bla* GripTite^{TM} cells, and the results are expressed as 17β-estradiol equivalent (E2-EQ) and 4-hydroxytamoxifen equivalent (4-OHTMX-EQ). E2-EQ estrogenic activity of the influent ranged between 9.7 and 13.9 ng/L (n=2) but was observed to reduce after the treatments with laccase alone and laccase/PS system (**Table 6.2**). Importantly, laccase-catalysed degradation achieved complete elimination of E2-EQ estrogenic activity (*i.e.,* below the detection limit of 1.4 ng/L). Despite the increase in toxicity, reduction in E2-EQ estrogenic activity in laccase/PS system (**Table 6.2**) demonstrate that the resultant transformation by-products either are not capable of exhibiting estrogenicity or have lower estrogenic activity than the parent compound. Regardless of the treatment option, the 4-OHTMX-EQ (*i.e.,* antiestrogenic activity) of the influent and the treated effluent was below the limit of detection (*i.e.,* less than 20 µg/L) as indicated in **Table 6.2**. Although laccase [41, 47] or PS [48, 49] treatments have been reported to significantly reduce estrogenic activity caused by TrOCs, the combination of both laccase and PS is also effective in reducing estrogenic activity.

6.4.2. Continuous TrOC removal by laccase/PS-NFBR treatment system

The laccase/PS-NFBR system was operated continuously for a period of 64 h (*i.e.,* 4 ×HRT) at the TrOC loading rate of 0.72 mg/L.d, and laccase activity of 90-95 $\mu M_{(DMP)}$/min. PS concentration of 5 mM was selected based on the performance of batch laccase/PS system, and was added only once at the start of the experiment. Main reason for coupling a membrane with an enzymatic bioreactor was to effectively retain laccase, thereby preventing laccase washout with treated effluent [14, 15]. To confirm this, permeate samples were collected after regular

intervals for monitoring the residual laccase activity as well as PS concentration. In this experiment, the NF membrane effectively retained laccase, and PS was also not detected in membrane permeate (data not shown). PS concentration may deplete over time due to the interaction of secondary radicals with sulphate radicals as explained in the **Section 6.4.1.2**, requiring intermittent addition of PS. Samples were collected after every 12 h for monitoring PS concentration in the laccase/PS bioreactor. A gradual reduction in PS concentration was observed during the operation of the laccase/PS-NFBR system. PS was not completely depleted by the end of continuous experiment (*i.e.*, after 64 h), and total reduction in PS concentration was approximately 55%. In the following section, degradation and overall removal of TrOCs in laccase/PS-NFBR is discussed separately for elucidating the performance of the developed treatment system.

6.4.2.1. TrOC degradation

In the continuous-flow laccase/PS-NFBR treatment system, mechanisms of TrOC removal include degradation by laccase/PS and membrane retention (discussed in **Section 6.4.2.2**). TrOC degradation by the laccase/PS during continuous treatment ranged between 56 and 100% (**Figure 6.6a**) with >99% degradation achieved for one non-phenolic (diclofenac) and two phenolic TrOCs (bisphenol A and oxybenzone). Although the comparison of data obtained from batch and continuous experiments may not be appropriate, better degradation of TrOC was observed in the laccase/PS-NFBR system as compared to that achieved in the batch laccase/PS system (**Figure 6.1**). Difference in the degradation of TrOCs in batch enzymatic bioreactor and continuous-flow EMBR was also reported by Nguyen et al. [13]. They reported that the degradation of a few TrOCs such as oxybenzone and diclofenac improved significantly by 10 to 60% in a continuous-flow EMBR as compared to the batch enzymatic bioreactor [13]. Indeed, in the current experiment, degradation of carbamazepine, sulfamethoxazole, and diclofenac by the laccase/PS-NFBR system was 52, 60 and 100%, respectively (**Figure 6.6b**), while their degradation in batch tests under an identical PS concentration of 5 mM was less than 55% (**Figure 6.1**). Previously, simultaneous retention of both laccase and TrOCs has been reported to facilitate degradation [33, 50]. In a study by Asif et al. [5], performance of an enzymatic bioreactor coupled to either an NF or UF membrane was compared under identical operating conditions. Compared to UF-EMBR, degradation of the selected TrOCs, namely carbamazepine, sulfamethoxazole, diclofenac, atrazine and oxybenzone improved by 15 to30% in NF-EMBR, and it was attributed to the prolonged contact time between laccase and TrOCs following their complete retention by the NF membrane [5].

It is important to note that the presence of TrOCs containing a phenolic moiety can also facilitate the degradation of non-phenolic pollutants by acting as a redox-mediator. In natural environmental settings, laccase can only degrade the phenolic components of lignin. This results in the formation of secondary radicals (*e.g.*, phenoxyl radical) or cross-coupling agents that can degrade the non-phenolic components of lignin [32]. In a recent study, the transformation by-products of a phenolic pharmaceutical (acetaminophen) was reported to directly oxidize another pharmaceutical carbamazepine [51]. It is possible that the phenolic TrOCs (oxybenzone and bisphenol A) that were effectively eliminated (~100%) may have

contributed in the degradation of non-phenolic TrOCs. This phenomenon may not be apparent in batch experiments probably because the abundance of oxidative cross coupling agents or secondary radicals was not high enough as compared to that in the continuous-flow laccase/PS-NFBR system. Since the NF membrane can achieve effective retention of laccase, PS and TrOCs, the transformation by-products will also stay in the bioreactor of the continuous-flow laccase/PS-NFBR system to contribute in TrOC degradation *via* catalytic and non-catalytic pathways.

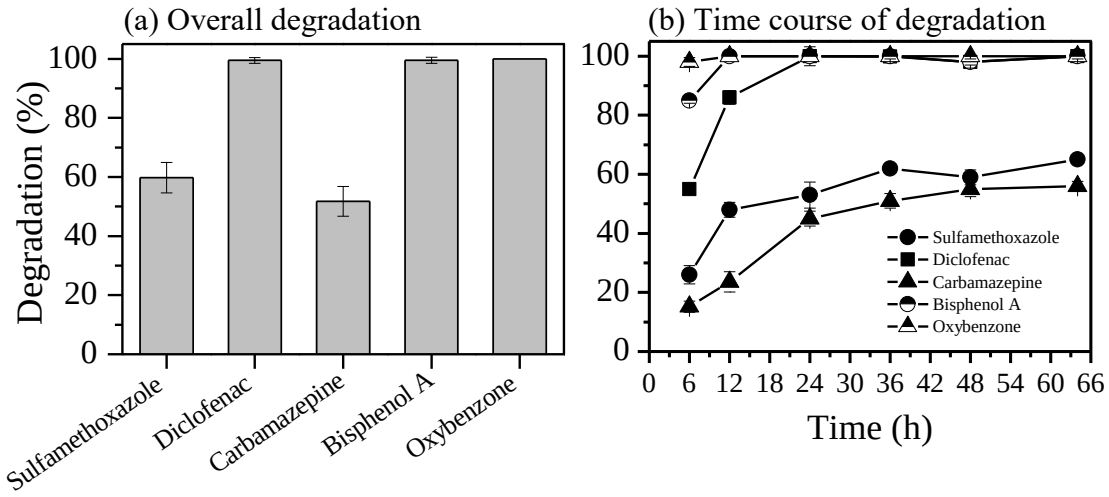


Figure 6.6. Performance of the laccase/PS-NFBR treatment system for the degradation of the selected TrOCs. Overall TrOC degradation (a) as well as time course of TrOC degradation (b) is shown here. PS (potassium persulfate) was added at 5 mM concentration, while the initial laccase activity was 90-95 $\mu M_{(DMP)}$/min. The laccase/PS-NF treatment system was operated at a TrOC loading rate of 0.72 mg/L.d and HRT of 16 h. Results presented as average ± standard-deviation calculated based on the triplicate samples that were collected at 24, 36, 48 and 64 h.

The performance of the laccase/PS-NFBR system was regularly monitored over the course of its operation for assessing the stability of the developed process. The time course of TrOC degradation during continuous operation as presented in **Figure 6.6(b)** indicates that the degradation of both phenolic TrOCs (bisphenol A and oxybenzone) stabilised after an operating time of 12 h, while steady state of diclofenac degradation was achieved after 18 h. Degradation of sulfamethoxazole and carbamazepine increased up until 36 h, and remained almost constant during the operating time of 36-64 h. Thus, it could be concluded that the developed system was stable, achieving steady-state TrOC degradation.

6.4.2.2. Overall TrOC removal

The benefits of integrating the laccase/PS system with a high retention NF membrane can only be realised by assessing the overall TrOC removal (*i.e.*, degradation + membrane retention). Despite the appreciable TrOC degradation, sulfamethoxazole and carbamazepine were resistant to laccase/PS system and were moderately degraded (52-60%). The NF membrane effectively retained the TrOCs not completely degraded by laccase/PS, producing a TrOC-free stream with >95% overall removal (**Figure 6.7**). The NF membrane can effectively retain a wide range of TrOCs *via* a combination of removal mechanisms. TrOC with molecular weight

above 200 g/mol has been reported to be effectively retained (above 90%) by size exclusion. On the other hand, mechanism of removal for hydrophobic (log D>3) and charged (*e.g.,* diclofenac) TrOCs is adsorption on membrane surface and charge repulsion, respectively [52, 53]. In a previous study, the NF membrane coupled to an enzymatic bioreactor achieved an overall removal of 92 to99% [5]. In this study, overall removal of the selected TrOCs ranged between 95 and 100%. Since molecular weight of all the selected TrOCs was above 200 g/mol (*see* **Table 6.1**), size exclusion appears to be the dominant mechanism of TrOC retention by the NF membrane.

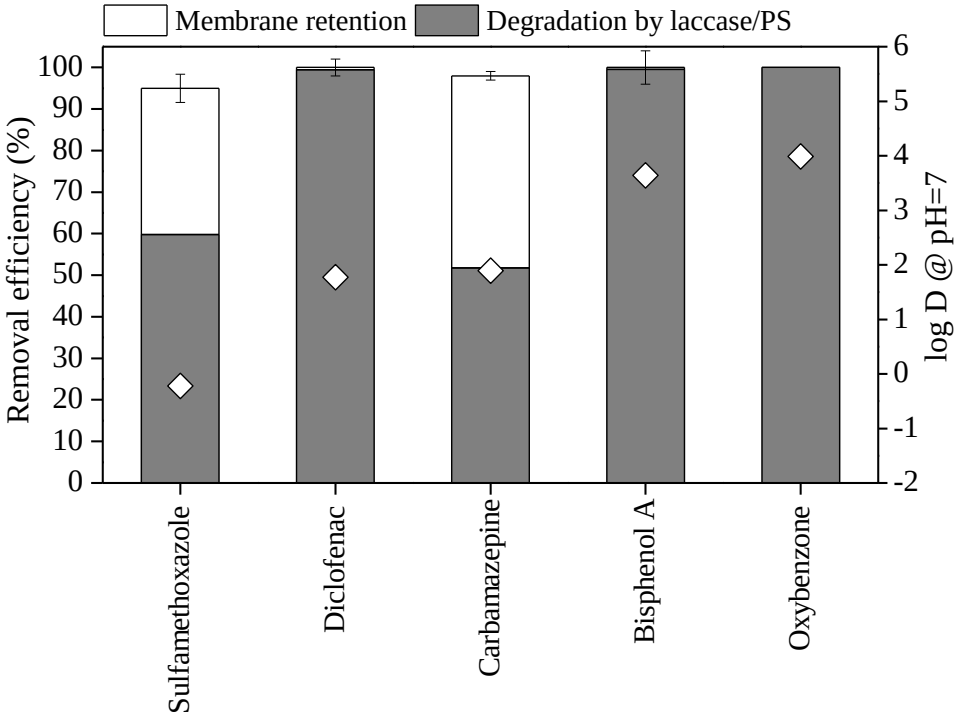


Figure 6.7. Overall removal (degradation + membrane retention) of TrOCs by the laccase/PS-NFBR system. Error bars represents the standard deviation (n=12). Experimental conditions are presented in the caption of **Figure 6.6**.

As demonstrated above in **section 6.4.1.4**, toxicity of the treated effluent increased after the batch laccase/PS treatment. In a previous study, a high retention membrane distillation process coupled to an enzymatic bioreactor was reported to reject TrOCs and their transformation products, thus producing a non-toxic permeate [50]. However, toxicity of the NF permeate has not been evaluated to date. To confirm this, samples were collected at the end of continuous operation, and their toxicity was analysed. Although the toxicity of the bioreactor media was 96.2 ± 6.7 rTU (n=3), NF permeate samples were non-toxic (*i.e.,* less than 1 rTU). This confirmed that the high retention NF membrane not only retained TrOCs but can also retain the transformation by-products that may exhibit toxicity.

Estrogenic activity is another important parameter to evaluate the safety of treated effluent for disposal and reuse [29, 54]. The samples collected from laccase/PS bioreactor at the end of experiment showed an estrogenic activity of 0.6 ± 0.2 ng/L EE2-EQ, while no estrogenic activity was found in NF permeate (*i.e.,* blow the detection limit of 0.35 ng/L EE2-EQ).

Importantly, the antiestrogenic activity expressed as 4-OHTMX-EQ in both the laccase/PS bioreactor and NF permeate was below the detection limit of 20 µg/L.

6.4.3. Hydraulic performance of the laccase/PS-NFBR

Permeate flux was monitored regularly for analysing the hydraulic performance of the NF membrane. By the end of the experiment, permeate flux of the NF membrane reduced by almost 20% (**Figure 6.8**). The permeate flux reduced by 10% within the first 2 h of operation due to the adsorption of the reaction media consisting of TrOCs, laccase and PS. Similar trend was observed previously when an enzymatic bioreactor was integrated with the NF membrane [5, 55]. Notably, the reduction in permeate flux can also be attributed to concentration polarization that is caused by the accumulation of the bioreactor media on or near the surface membrane [55, 56]. At the end of operation, cleaning the NF membrane with milli-Q water was effective to recover the flux by almost 95%. This indicates that the reduction in flux caused due to the adsorption of reaction media and/or concentration polarization is reversible.

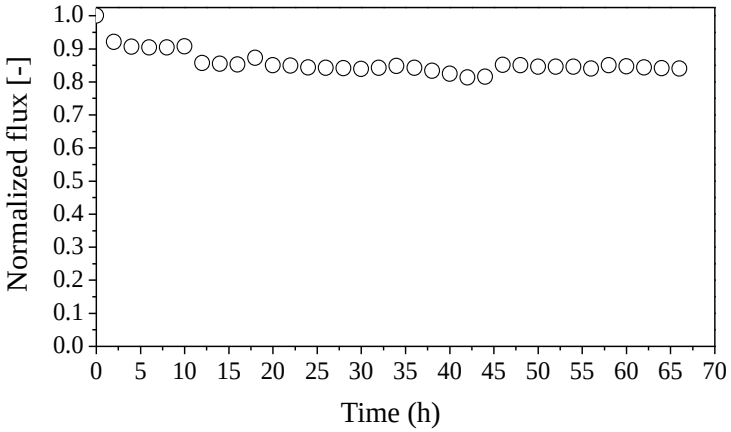


Figure 6.8. Hydraulic performance of the NF membrane expressed as normalised flux during the operation of the laccase/PS-NFBR treatment system.

6.5. Conclusion

Performance of an integrated laccase and persulfate (PS) oxidation process in batch and continuous modes was systematically investigated for the degradation of five trace organic contaminants (TrOCs), namely diclofenac, sulfamethoxazole, carbamazepine, bisphenol A and oxybenzone. Degradation of the selected TrOCs by laccase alone in batch experiments ranged between 7 (for sulfamethoxazole) and 100% (for oxybenzone) and was governed by the physicochemical properties such as chemical structure. Addition of PS at different concentration (1-10 mM) in the batch enzymatic bioreactor achieved TrOC-specific improvement in degradation, exhibiting the benefits of combining both oxidation processes. Among the tested PS concentrations (1-10 mM), the best performance was achieved at 5 mM concentration that achieved 100% degradation for two phenolics (bisphenol A and oxybenzone) and 25-53% degradation for three non-phenolic (diclofenac, sulfamethoxazole, and carbamazepine) TrOCs without significantly causing laccase inactivation and PS depletion. However, addition of PS increased the toxicity of the treated effluent to 7.6-28.8 rTU as compared to the toxicity of <1-2.3 rTU obtained after treatment with laccase alone. All

treatment options with and without PS reduced the estrogenic activity measured as 17β-estradiol equivalent, while antiestrogenic activity (expressed as 4-hydroxytamoxifen equivalent) was below the detection limit of 20 µg/L. A treatment system by coupling a nanofiltration (NF) membrane with laccase/PS (5 mM) system was also developed, which allowed continuous removal of TrOCs without laccase and PS washout. The continuous laccase/PS-NFBR treatment system produced a high quality permeate stream due to effective TrOC removal (95-100%). In addition, the enhanced degradation (10-65%) of non-phenolic was also achieved in laccase/PS-NFBR as compared to that obtained in the batch laccase/PS system. On the other hand, degradation of both bisphenol A and oxybenzone remained below the limit of detection in the laccase/PS-NFBR system. Although the toxicity of the bioreactor reaction media increased, the membrane permeate was non-toxic. The evaluation of the data obtained from estrogenic activity assay indicated a reduction in the estrogenic activity of the reaction media. Because the NF membrane can reject the residual estrogenic activity, the membrane permeate was deemed safe for disposal and reuse. Hydraulic performance of the NF membrane was monitored, which showed a reduction of approximately 20% in the permeate flux at the end of the experiment. Cleaning the membrane with Milli-Q water helped to recover permeate flux by 95%.

6.6. References

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Chapter 7: Simultaneous removal of TrOCs and metals by a PS-assisted membrane distillation reactor

This chapter is based on the following publication:

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7.1. Introduction

Acid mine drainage (AMD) originating from different mining activities (such as coal and gold mining) has gained global attention in the last decade due to its adverse environmental impacts on soil, freshwater and aquatic ecosystem [1, 2]. AMD is generated due to the oxidation of sulfides ores during a wide range of mining activities. Among different types of sulfide ores, pyrite ore (FeS_2) has been recognized as the main mineral ore for AMD generation, because its exposure to oxygen and water can easily oxidize the pyrite ore as shown in Equation (1-3) [1, 3]. Composition of AMD is diverse and is commonly characterized by high concentrations of sulphate and iron (II) as well as other trace elements such as calcium and lithium. AMD flows into freshwater bodies and can promote the release of different toxic metals following its interaction with underground rocks [1, 4, 5]. Thus, an efficient process for the treatment of AMD-impacted water is required for maintaining high water quality.

$$FeS_2 + \frac{7}{2}O_2 + H_2O \rightarrow Fe^{2+} + 2{SO_4}^{2-} + 2\mathrm{H}^+ \quad \text{Eq. (1)}$$

$$Fe^{2+} + \frac{1}{4}O_2 + \mathrm{H}^+ \rightarrow Fe^{3+} + \frac{1}{2}H_2O \quad \text{Eq. (2)}$$

$$Fe^{3+} + 3H_2O \rightarrow Fe(OH)_3 + 3\mathrm{H}^+ \quad \text{Eq. (3)}$$

Technologies for the treatment of AMD-impacted water can be divided into two categories, namely active and passive treatment processes. The conventional active treatment process includes the application of alkaline chemicals (*e.g.*, lime) to precipitate metals by raising the pH [6]. However, this process generates toxic sludge that contains high concentrations of amorphous ferric oxyhydroxide, and the treated effluent requires additional treatment for meeting the water quality guidelines [1, 6]. Bioreactors and wetlands are the passive processes, which use natural processes for the treatment AMD-impacted water. For instance, during treatment with bioreactors, naturally occurring iron and sulfate reducing bacteria are cultured to convert sulfate into hydrogen sulfide, but the pH of AMD-impacted water should be above 3 for effective metabolization of iron and sulfate ions [7]. During treatment with wetlands, natural attenuation processes, lime dosing, and long hydraulic retention time (HRT) allow all the solids including heavy metals to settle down [8]. Although conventional active and passive processes are effective, large area requirement; large quantities of reagents for neutralization; and long HRT as well as process sensitivity to changes in influent chemistry (such as pH and temperature) are the major drawbacks [9-11]. Hence, a compact and robust treatment process is required for the treatment of AMD-impacted water.

In addition to AMD contamination, trace organic contamination (TrOCs) such as pharmaceuticals and pesticides have been reported to be detected in sewage-impacted freshwater bodies [12-14]. This raises significant concern due to their potential harmful impact on aquatic organisms and even humans in the case of prolonged ingestion, thus requiring a treatment process for effective removal of both the metals and TrOCs. Recently membrane distillation (MD) has gained significant attention as an effective separation process [15, 16].

MD is a thermally driven membrane separation process; however, it requires a lower operating temperature than conventional distillation processes such as fractional distillation. During the MD process, water in vapor form moves *via* diffusion through a microporous hydrophobic membrane from a higher temperature feed solution to a lower temperature permeate solution. This occurs due to the vapor pressure gradient developed by the temperature difference between the sides of the membrane [17, 18]. Since the water moves across the membrane in vapor form, MD can theoretically provide complete removal of non-volatile pollutants [15, 19]. Furthermore, the compatibility of the MD process with low-grade waste heat and solar thermal energy [17] makes its application attractive in various fields, including water desalination and wastewater treatment.

The MD process has been predominantly assessed for the desalination of sea and brackish water, particularly for hyper-saline feed, because, being a thermally driven process, water flux in MD is negligibly affected by the feed osmotic pressure as compared to the pressure-driven membrane desalination processes (*e.g.*, RO and NF) [16, 20, 21]. In addition, as compared to RO and NF membranes, superior performance of MD for the removal of a range of pollutants such as pharmaceuticals and metal salts has been reported [22-25]. Despite the potential to date, the performance of MD for the removal of TrOCs and metals has been mostly assessed separately only in a few short-term batch studies. For instance, Wijekoon et al. [15] investigated the removal of TrOCs including pesticides and pharmaceuticals by MD operated in batch mode for 24h. They observed TrOCs removal to be governed by their volatility and hydrophobicity. In another short-term study by Han et al. [22], MD achieved 90-95% removal of a nonsteroidal anti-inflammatory drug ibuprofen from synthetic wastewaters mimicking surface water or reverse-osmosis concentrate. Hull and Zodrow [26] investigated the removal of AMD constituents including iron, aluminum and zinc from synthetic by using an MD process. They achieved above 99% removal of AMD constituents by MD after a short-term experiment of 12-24 h [26]. Although these studies provide useful insights, it is important to note that a continuous-flow operation is required to analyze and understand process stability. To date, only a handful of studies have assessed TrOC removal in continuous-flow mode [16, 27]. The authors reported 70 to 99% removal of the investigated TrOCs depending on their physicochemical properties. Two particular aspects highlighted in these studies were: (a) membrane fouling, significantly reducing permeate flux; and (b) additional requirement of treatment and disposal of membrane-concentrate rich in TrOCs as well as other organic and inorganic impurities. Notably, a continuous-flow MD process for the treatment of AMD-impacted water is yet to be studied.

Accumulation of organic pollutants, particularly TrOCs in MD-concentrate could be reduced by integrating either a laccase or a persulfate (PS) oxidation process with the MD process. This integration will simultaneously degrade bulk organics and TrOCs, reducing their accumulation in MD-concentrate and potentially mitigating membrane fouling. Efficacy of laccase-catalyzed

degradation process and PS-assisted oxidation processes for TrOC removal has already been discussed in **Chapter 6**.

In this chapter, the performance of a direct-contact MD process is discussed for simultaneous removal of metal salts and TrOCs from sewage- and AMD-impacted water. For this purpose, a synthetic wastewater was prepared, which contains a mixture of 12 TrOCs at an environmentally relevant concentration of 5 µg/L, as well as a mixture of four metal ions (iron, calcium, magnesium and lithium each at 10 and 100 mg/L concentration). The performance of laccase and PS for the degradation of TrOCs was systematically analyzed, and the oxidation process showing better stability and TrOC removal was selected for integration with the MD. Basic water quality parameters such as total organic carbon (TOC) and total nitrogen (TN) as well as membrane water productivity was thoroughly evaluated to determine the fouling behavior. At the end of operation, MD membranes were characterized by scanning electron microscopy (SEM) - energy dispersion spectrometry (EDS) to gain an in-depth understanding of the fouling mitigation.

7.2. Hypothesis

- The direct-contact MD process can achieve effective removal of TrOCs and metal salts from sewage- and AMD-impacted water
- Accumulation of organic and inorganic impurities (such as metal ions) in membrane-concentrate may affect the hydraulic performance of the MD process by causing membrane fouling
- Integration of an oxidation process with MD may achieve TOC and TrOC degradation as well as membrane fouling control

7.3. Materials and methods

7.3.1. Chemicals

Potassium persulfate (PS) was purchased from Sigma-Aldrich (Australia). The stock solution (100 mM) of PS was prepared in ultrapure Milli-Q water and stored at 4ºC before use. HPLC grade acetonitrile, methanol, dichloromethane and formic acid were used for quantification of TrOCs as explained in **Section 7.3.4.2**. As noted in **Section 7.3.3.2**, analytical grade glucose, peptone, urea, monopotassium phosphate, magnesium sulphate, ferrous sulphate, and sodium acetate were used to make the synthetic wastewater for MBR. In this experiment, metal salts and TrOCs were added in the MBR permeate for making the synthetic wastewater. MBR permeate was used to mimic sewage-impacted water.

Properties of the selected TrOCs including three pesticides and nine PPCPs are presented in **Table 7.1**, and their chemical structures are given in **Appendix Table 7-1**. These were selected based on their widespread occurrence in municipal wastewater and sewage-impacted water [12]. These chemicals were also purchased from Sigma-Aldrich (Australia). A combined stock solution of TrOCs was prepared in pure methanol and stored at −18ºC in dark. Analytical grade

iron sulfate, lithium chloride, magnesium chloride and calcium chloride were purchased from Sigma-Aldrich (Australia), and a concentrated stock solution containing the mixture of each metal salt was prepared in 100 mL Milli-Q ultrapure water. Laccase from genetically modified *Aspergillus oryzae* obtained from Novozymes Pty Ltd (Australia) was used in this experiment. Properties of laccase are presented in **Section 3.3.1 (Chapter 3)**.

Table 7.1. Physicochemical properties of the selected TrOCs

Type	Name	Molecular weight [a] (g/mol)	log D @ pH=7 [a]	pK_a [a]	pK_H @ pH=7 [b]	Charge at pH=7
Pharmaceuticals and personal care products (PPCPs)	Acetaminophen	152	0.46	0.52	8.3	Negative
	Bezafibrate	362	-0.93	3.29	-	
	Diclofenac	296	1.77	4.18	11.51	
	Sulfamethoxazole	253	-0.96	5.18	11.81	
	Amitriptyline	277	2.28	9.18	8.18	Neutral
	Carbamazepine	236	1.89	13.94	9.09	
	Primidone	218	0.83	12.26	13.93	
	Triclosan	290	5.28	7.8	6.18	
	Trimethoprim	290	0.27	7.04	13.62	
Pesticide	Atrazine	216	2.64	2.27	7.28	Negative
	Linuron	249	3.12	12.13	8.71	
	Pentachlorophenol	266	2.85	4.68	7.59	Neutral

[a] molecular weight, log D (water partition coefficient) and pk_a (acid dissociation coefficient) were obtained from the SciFinder Scholar database

[b] $pK_H = - \log_{10} H$, where H is Henry's law constant and defined as vapour pressure×molecular weight/water solubility.

"–": not available

7.3.2. Experimental setup

A laboratory-scale direct contact membrane distillation (DCMD) system was used for the treatment of synthetic sewage- and AMD-impacted water (**Figure 7.1**), due to the ease of operation as compared to other MD configurations, *e.g.,* air gap MD [17]. **In Chapter 4 and 5**, the MD setup was operated in concentration mode, and is different from that used in this experiment. Hence, a brief description of the continuous-flow MD setup is provided here. The DCMD setup consisted of a 3 L glass reactor (hereafter referred to as MD feed tank), a membrane module, a glass distillate tank (5 L) and two circulation gear pumps (Micropump Inc., Vancouver, WA, USA). Operated *via* a water level controller, a peristaltic pump (Cole-Parmer, Vernon Hills, IL, USA) supplied wastewater from a storage tank to the MD feed tank. The temperature of the MD feed tank, which was covered, was maintained at 40 ± 1.5ºC by using a heating immersion circulator (Julabo, Seelbach, Germany), while a chiller (Thermo Scientific, Waltham, MA, USA) was used to keep the temperature of the distillate tank at 20 ± 0.5ºC.

The MD membrane module was made of acrylic plastic. It was comprised of two identical cells, each engraved with flow channels 145 mm long, 95 mm wide and 3 mm deep as described previously [28]. A hydrophobic polytetrafluoroethylene (PTFE) membrane with a thickness, nominal pore size, and porosity of 60 μm, 0.2 μm, and 80%, respectively, was purchased from

Ningbo Porous Membrane Technology (Ningbo, China). The media from the MD feed tank and the distillate tank were passed through the opposite membrane cells at a recirculation flowrate of 1 L/min (corresponding to a cross flow velocity of 9 cm/s) using two rotameters. The partial vapor pressure gradient developed due to difference in temperature allows water to move across the membrane as vapor, consequently increasing the volume of water in distillate tank. This tank was placed on a precision balance (Mettler-Toledo, Kings Park, NSW, Australia). Change in the weight of distillate water was recorded in a computer *via* BalanceLink software (Mettler Toledo) to determine the MD water flux.

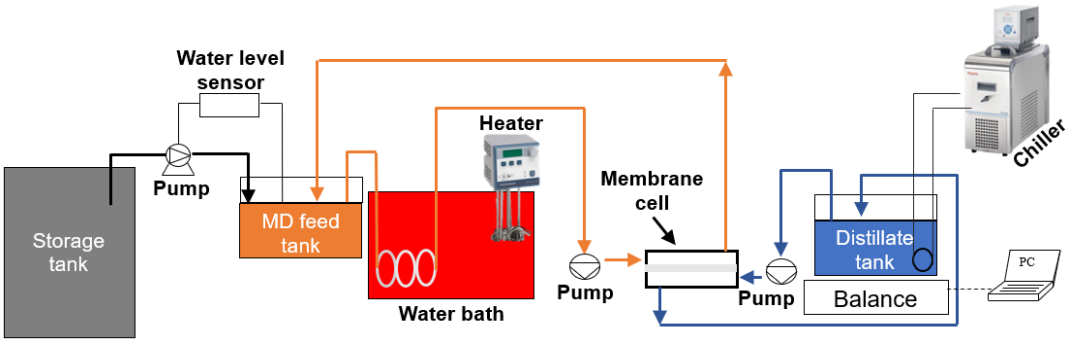

Figure 7.1. Schematic representation of the laboratory-scale DCMD setup

7.3.3. Experimental protocols

7.3.3.1. MD process characterization

The MD process was characterized by calculating the mass transfer coefficient (K_m) using a procedure previously described by Duong et al. [18]. Briefly, the MD system was operated in batch mode at different feed temperatures (*i.e.*, 40, 45 and 50 ºC) for 1 h with ultrapure Milli Q water. Distillate temperature was kept constant at 20 ºC, and recirculation flow rate of both feed and distillate was maintained at 1 L/min. The permeate flux was recorded every 5 min for 1 h. Permeate flux of MD can be theoretically calculated using Equation 4 as given below:

$$J = K_m \times (P_{feed} - P_{distillate}) \quad \text{Eq. (4)}$$

where J is the permeate flux (L/m^2 h) of DCMD, K_m is the mass transfer coefficient (L/m^2 h Pa), P_{feed} is the vapor pressure of water in MD feed, and $P_{distillate}$ is the vapor pressure of water in MD distillate. P_{feed} and $P_{distillate}$ can be determined by using Equation 5 [29]:

$$P = x_{water} \times \alpha_{water} \times P_o \quad \text{Eq. (5)}$$

where x_{water} and α_{water} are the molar fraction and activity of water, respectively, and P_o is the vapor pressure of water in MD feed and distillate. Since DCMD was characterised with ultrapure Milli-Q water, value of both x_{water} and α_{water} is equal to 1. Vapor pressure of water in MD feed and distillate can be calculated by using Antoine's Equation [18, 29] as given below:

$$P_o = \exp(23.1964 - \frac{3816.44}{T - 46.13}) \quad \text{Eq. (6)}$$

where T is the absolute temperature of the feed or distillate streams.

7.3.3.2. Performance of a stand-alone MD process

In the first part of this experiment, removal of both the TrOCs and metalions from four different compositions of synthetic wastewater was studied by operating the stand-alone MD in a continuous-flow mode for a period of 5 d, *i.e.*, 6×HRT (**Table 7.2**). The main aim was to understand the interaction of different pollutants on their rejection as well to understand the performance governing factors.

Table 7.2. Description of different wastewater compositions treated by the stand-alone MD

Run ID	Milli-Q water	MBR permeate	TrOCs	Metal salts
Control run	✓	–	✓	✓
MD-WW_0	–	✓	✓	–
MD-WW_{10}	–	✓	✓	✓
MD-WW_{100}	–	✓	✓	✓

Note: TrOC concentration was kept at 5 µg/L during all experiments.
The numbers in the sub-script of the run ID indicate the concentration of each metals salt. For example, MD-WW_0 represents the run with no metal salts, while MD-WW_{10} indicates that the concentration of each salt during this experiment was 10 mg/L.

As mentioned above, permeate from a lab-scale MBR was collected to make a synthetic wastewater mimicking the properties of sewage- and AMD-impacted water. The MBR was operated for around one year, while it was continuously fed with synthetic wastewater containing 400 mg/L glucose, 100 mg/L peptone, 35 mg/L urea, 17.5 mg/L monopotassium phosphate, 17.5 mg/L magnesium sulphate, 10 mg/L ferrous sulphate, and 225 mg/L sodium acetate. The wastewater had a chemical oxygen demand (COD), total organic carbon (TOC), total nitrogen (TN) and PO_4^{3-}-P concentrations of 650, 175, 25, and 15 mg/L, respectively. The hydraulic retention time and solids retention time of the MBR was 12 h and 10 d, respectively. Characteristics of MBR permeate are given in **Table 7.3**.

Table 7.3. Characteristics of MBR permeate used for making the different compositions of wastewater to be treated by MD

Parameter	Unit	Value (minimum – maximum)
pH	-	6.9 – 7.2
Conductivity	µS/cm	190 – 220
Total organic carbon (TOC)	mg/L	8.5 – 16
Total nitrogen (TN)	mg/L	6.4 – 7.9
NH_4^+-N	mg/L	2.4 – 2.9
PO_4^{3-}-P	mg/L	3.4 – 6.1

Prior to the commencement of this experiment, the MBR-treated effluent was spiked with the selected pharmaceuticals and pesticides (at 5 µg/L each) as well as metal salts at either 10 or 100 mg/L each as illustrated in **Table 7.2**. Duplicate samples from MD feed tank and distillate

tank were collected after every 3×HRT for the quantification of TrOCs and metal ions. In addition, samples were collected on daily basis to measure TOC and TN removal by the MD. At the start of each run, 1.5 L of Milli-Q was added in the distillate tank that served as the initial distillate. Thus, the concentrations of TOC, TN, TrOCs and metals in MD permeate were corrected for dilution by considering the initial working volume of the distillate tank. At the end of MD operation, MD membranes were collected and characterized by SEM-EDS to gain an in-depth understanding of the fouling constituents.

7.3.3.3. Performance of an integrated MD process

To choose an oxidation process to be integrated with MD, performance and stability of both laccase and PS was assessed in batch experiments. The batch reactors (250 mL) containing the wastewater prepared by adding the mixture of TrOCs and iron salt in MBR permeate were incubated for a period of 24 h at 20 and 40 °C in separate runs. Initial laccase activity and PS concentration was maintained at 95-100 µM/min and 1 mM, respectively. Samples were collected at the end for TrOC quantification, laccase activity and residual PS concentration.

Based on the results achieved (*see* **Section 7.4.3.1**), PS exhibited better TrOC removal and stability in batch experiments as compared to laccase. Thus, PS-assisted oxidation process was selected for integration with MD. PS was directly added to the feed media at a concentration of 1 mM after every 2×HRT. Concentration of PS was selected based on a comprehensive literature survey [30, 31]. The spiked secondary treated wastewater was treated by PS-assisted MD system with (PS-MD-WW_{10}) and without (PS-MD-WW_0) the addition of metal salts in continuous-flow mode for a period of 5 d (*i.e.*, 6×HRT).

Duplicate samples from MD feed tank and distillate tank were collected after every 3×HRT for the determination of TrOCs and metals. In addition, samples were collected on daily basis to measure TOC and TN removal by PS-assisted MD with and without the addition of metal salts. Similar to the stand-alone MD, membranes at the end of PS-assisted MD systems were collected and characterized by SEM-EDS to gain an in-depth understanding of the fouling control achieved by PS.

7.3.4. Analytical methods

7.3.4.1. Analysis of basic quality parameters

Samples from MD feed tank and distillate tank were collected on daily basis for analysis. TOC and TN concentrations were measured using a TOC/TN-V_{CSH} analyser (Shimadzu, Japan). TOC and TN removal efficiency by the stand-alone and PS-assisted MD were calculated based on the method described in **Section 7.3.4.2**. The pH and conductivity were measured using an Orion 4 Star Plus portable pH/conductivity meter (Thermo Scientific, Waltham, MA, USA)

7.3.4.2. Analysis of TrOCs and metals

TrOCs were analysed using a Shimadzu LC-MS system (LC-MS 2020) after solid phase extraction (SPE). A detailed description of this method is available elsewhere [32]. Briefly, TrOCs were extracted using 6 mL Oasis HLB cartridges (Waters, Milford, MA, USA). The HLB cartridges were first pre-conditioned with 5 mL dichloromethane and methanol solution (1:1 v/v), 5 mL methanol and 5 mL Milli-Q water. The pH of the samples was adjusted to 2-3 using 2 M H_2SO_4, and then loaded onto the cartridges at a flow rate of 1–4 mL/min. The cartridges were dried for 30 min under gentle stream of nitrogen. The extracted samples were eluted using 7 mL methanol and 7 mL dichloromethane and were dried in a water bath at 40ºC for 3-4 h. The residues were redissolved in 400 µL methanol for quantification by LC-MS.

The LC-MS system was equipped with an electrospray ionization (ESI) interface, and a Phenomenex Kinetex C8 chromatography column (50 × 4.6 mm) was used for the separation of TrOCs. Milli-Q water buffered with 0.1% (v/v) formic acid, and HPLC grade acetonitrile was used as the mobile phase during the analysis. Mobile phase flow rate and sample injection volume were 0.5 mL/min and 10 µL, respectively. Quantification of acetaminophen, primidone, trimethoprim, sulfamethoxazole, carbamazepine, bezafibrate, atrazine, linuron, and amitriptyline was performed under ESI positive ionization $[M+H]^+$ mode, while ESI negative ionization $[M-H]^-$ mode was adopted for pentachlorophenol, diclofenac and triclosan [32]. During the analysis, detector voltage, desolvation line temperature and heating block temperature were kept constant at 0.9 kV, 250ºC, and 200ºC, respectively. The analysis was conducted in gradient elution mode as shown in **Appendix Table 7-2**. High purity nitrogen that acted as both the nebulizing and drying gas was supplied continuously at a flow rate of 1.5 and 10 L/min, respectively. The calibration curves were prepared by analyzing the known concentrations of analytes that ranged between 0.1 and 20 µg/L. The correlation coefficient of all the calibration curves was above 0.99.

Removal of TrOCs by PS/laccase (R_1) and MD (R_2) was calculated by using Equation (7) and (8), respectively:

$$R_1 = 100 \times \left(1 - \frac{C_{su}}{C_f}\right) \quad \text{Eq. (7)}$$

$$R_2 = 100 \times \left(1 - \frac{C_p}{C_f}\right) \quad \text{Eq. (8)}$$

where, C_f, C_{su} and C_p are the concentration (ng/L) of a specific pollutant in MD storage tank (**Figure 7.1**), MD feed and MD permeate, respectively.

The mass of a TrOCs degraded by PS during PS-assisted MD operation was calculated as follows:

$$C_f \times V_f = (C_{su} \times V_{su}) + (C_p \times V_p) + \text{Mass degraded by PS} \quad \text{Eq. (9)}$$

where, V_f, V_{su} and V_p represents the volume of wastewater, MD feed and permeate, respectively.

Concentrations of iron (II), calcium, magnesium and lithium were determined by inductively coupled plasma-optical emission spectroscopy (ICP-OES, 7500CS, Agilent Technologies, USA). A sample dilution was prepared out with 5% nitric acid. The linear regression coefficients for all calibration curves were higher than 0.99 for both elements. Prior to each batch of analyses, the ICP-OES was tuned by a using multi-element tuning solution.

7.3.4.3. Membrane characterization and toxicity of MD permeate

At the end of DCMD operation with and without PS dosing, MD membranes were collected and air-dried in a desiccator. MD membranes were then coated with an ultra-thin gold layer with a sputter coater (SPI Module, West Chester, PA, USA), and were characterized with a scanning electron microscopy (SEM) coupled with energy dispersion spectrometry (EDS) (JCM-600, JEOL, Tokyo, Japan). The method for toxicity assay has already been described in **Section 5.3.4.3** (**Chapter 5**)**.**

7.3.4.4. PS concentration and laccase activity

The change in PS concentration following its addition to the reaction media was monitored during batch experiments as well as during the operation of PS-assisted MD by using a previously developed spectrophotometric method [33]. Briefly, two solutions were prepared before measuring PS concentration. Solution-1 was the PS stock solution (100 mM). Solution-2 was prepared by dissolving 0.2 g $NaHCO_3$ and 4 g KI in 40 mL Milli-Q water, mixed well and allowed to equilibrate for 15 min. Portions of Solution-1 (*i.e.*, 0.1, 0.2, 0.4, 2 and 4 mL) were separately added to Solution-2 to achieve final PS concentration of 0.25, 0.5, 1, 5 and 10 mM. The standard solutions were incubated on a rotary shaker at 80 rpm for 2 h. Absorbance of the standard solutions was measured at a wavelength of 352 nm in 1 cm quartz cuvettes using a UV-visible spectrophotometer (DR6000, Hach, Loveland, CO, USA). The coefficient of determination (R^2) obtained by drawing the calibration curve was >0.98. For determining the concentration of PS during the operation of PS-assisted DCMD, 20 mL sample collected from MD feed was added to 40 mL Solution-2, and the resulting solution was incubated for 2 h before measuring its absorbance at 352 nm using a UV-visible spectrophotometer as described above. The concentration of the PS was corrected by multiplying it with the dilution factor of 3. Laccase activity was measured as described in **Section 3.3.4.2** (**Chapter 3**).

7.4. Results and discussions

7.4.1. Mass transfer coefficient (K_m) of MD

The mass transfer coefficient (K_m) of the MD system in the current experiment was determined experimentally using ultrapure Milli-Q water as feed following Equations 4−6. Mass transfer (denoted by K_m value) during MD operation can be affected by concentration and temperature polarization. Since concentration of salts in Milli-Q water is negligible, the effect of

concentration polarization on K_m could be ignored. Temperature polarization effect has been incorporated in Equations 4−6 for the determination of K_m. The significance of temperature polarization effect can be assessed by comparing K_m values at different feed temperatures [17, 18]. Despite the increase in permeate flux (**Figure 7.2a**), K_m reduced with the increase of MD feed temperature from 40 to 50ºC (**Figure 7.2**). This indicates that temperature polarization effects become severe at high feed temperature, which is consistent with the available literature [18, 34, 35]. Therefore, we operated the DCMD system at a feed temperature of 40ºC, resulting a K_m value 2.7 L/m^2.h.Pa.

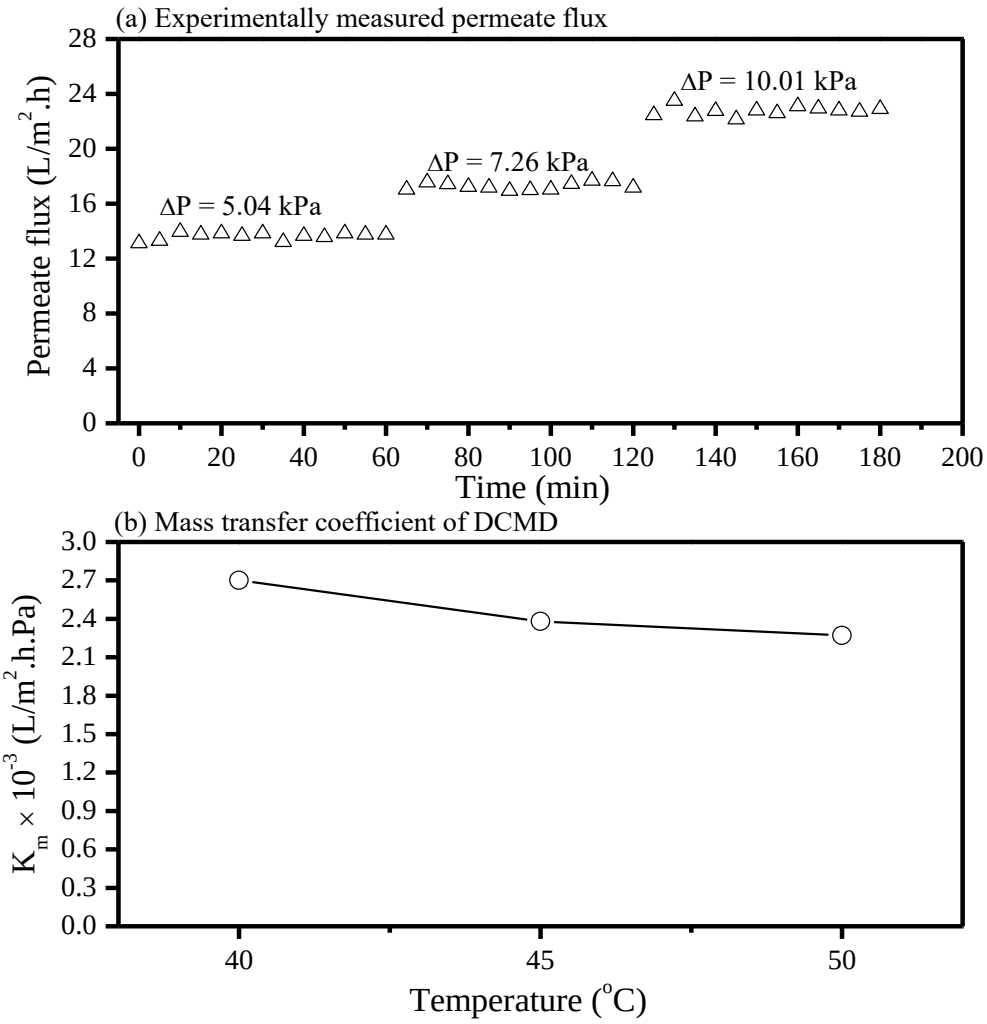

Figure 7.2. Permeate flux (a) and mass transfer coefficient (b) of the MD system determined experimentally with Milli-Q water as feed at a temperature of 40, 45 and 50 ºC. Temperature of the distillate reservoir was kept at 20 ºC, while the cross-flow velocity was maintained at 1 L/min.

7.4.2. Performance of a stand-alone MD system

7.4.2.1. Removal of TrOCs

Overall removal of the selected TrOCs by the standalone MD system in absence of metal salts is presented in **Figure 7.3**. In a stand-alone MD process, membrane retention is the only

mechanism of TrOC removal. Because water moves across an MD membrane in vapor form, the extent of TrOC removal by the membrane is influenced by the water partition coefficient (log D) and vapor pressure of the target pollutant [25]. Noting that $pK_H = -\log_{10} H$ (where, H is the Henry's Law constant and is equal to vapor pressure × MW/water solubility), in general, TrOCs with a low 'pK_H / log D' ratio (*e.g.*, less than 2.5) are partially removed by the MD membrane in a stand-alone MD system [15, 28]. In the current experiment, TrOC removal from two different compositions of wastewater (control and MD-WW_0) by MD was initially assessed. The stand-alone MD achieved TrOC-specific removal that ranged between 86 and 100% (**Figure 7.3**). During the control run, out of the 12 selected TrOCs, removal of six including four PPCPs (amitriptyline, acetaminophen, trimethoprim and triclosan) and two pesticides (pentachlorophenol and linuron) was between 90 and 98%, while removal of four PPCPs (primidone, bezafibrate, carbamazepine and sulfamethoxazole) was greater than 98% (**Figure 7.3**). For the remaining two TrOCs, removal of the pesticide atrazine and the nonsteroidal anti-inflammatory diclofenac was 85%.

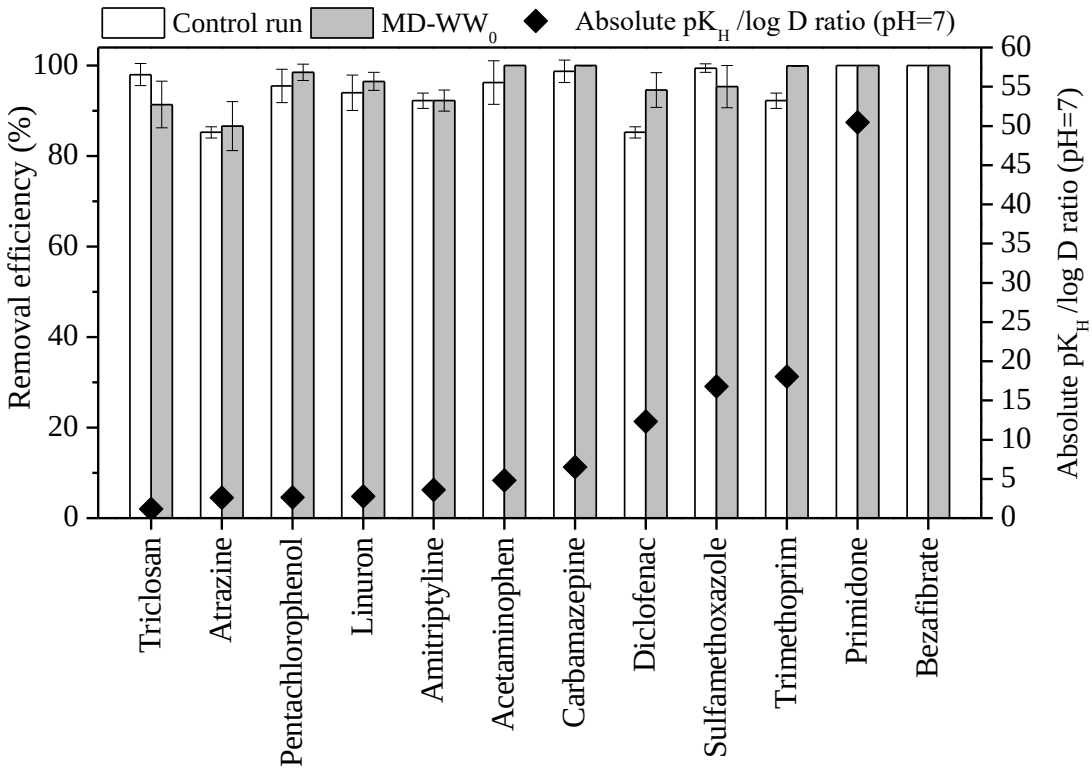

Figure 7.3. Performance of the stand-alone MD for the removal of the selected TrOCs arranged based on pK_H /log. Two different compositions of the synthetic wastewater were prepared to assess the performance of the stand-alone MD. For the control run, synthetic wastewater was prepared by adding a mixture of TrOCs in ultrapure Milli-Q water. For the MD-WW_0 run, synthetic wastewater was prepared by dosing MBR permeate with TrOC mixture. Operating conditions: the initial TrOC concentration was 5 µg/L; temperature of the MD feed and the distillate (permeate) tank was kept at 40 and 20 ºC, repectivley; and cross-flow rate was 1 L/min (corresponding to cross-flow velocity of 9 cm/s). Mean removal efficiency and standard deviation (n=4 for control run, and n=10 for MD-WW_0 run) are presented.

During the MD-WW_0 run for the treatment of wastewater prepared by dosing MBR permeate with TrOC mixture, six including four PPCPs (carbamazepine, trimethoprim, bezafibrate, primidone and acetaminophen) and three pesticide (pentachlorophenol) exhibited removal greater than 98% (**Figure 7.3**). For the remaining TrOCs, MD achieved a removal of 86% for atrazine, 91% for triclosan, 92% for amitriptyline, 94% for diclofenac, 95% for sulfamethoxazole, and 96% for linuron (**Figure 7.3**). Previously, Song et al. [27] investigated the performance of a stand-alone MD system for the treatment of anaerobic-MBR permeate containing a mixture of TrOCs. Consistent with the results of the current experiment, they also reported good but incomplete removal (80-95%) of a few TrOCs such as atrazine, diclofenac, sulfamethoxazole, linuron and triclosan [27]. Due to the adsorption of a few TrOCs onto the residual organics present in MBR permeate, TrOC removal from MBR permeate could be better than that achieved from milli-Q water [15]. Indeed, comparison of the results indicates that removal of two TrOCs (diclofenac and sulfamethoxazole) by the stand-alone MD was significantly better (up to 10%) during the MD-WW_0 run as compared to that during control run (**Figure 7.3**).

When the salts of iron, calcium, magnesium and lithium were added at either 10 or 100 mg/L in the MBR-permeate, no apparent change in the extent of TrOC removal by the stand-alone MD system was observed. TrOC removal ranged from 84 to 100% at a salt concentration of 10 mg/L each, and 82 to 100% at a salt concentration of 100 mg/L each (**Figure 7.4**). Comparison of the results obtained from this experiment could not be compared with the available literature due to unviability of the studies on removal of TrOCs in presence of metal salts. These results suggest that the stand-alone MD can achieve effective TrOC removal even in presence of metal salts.

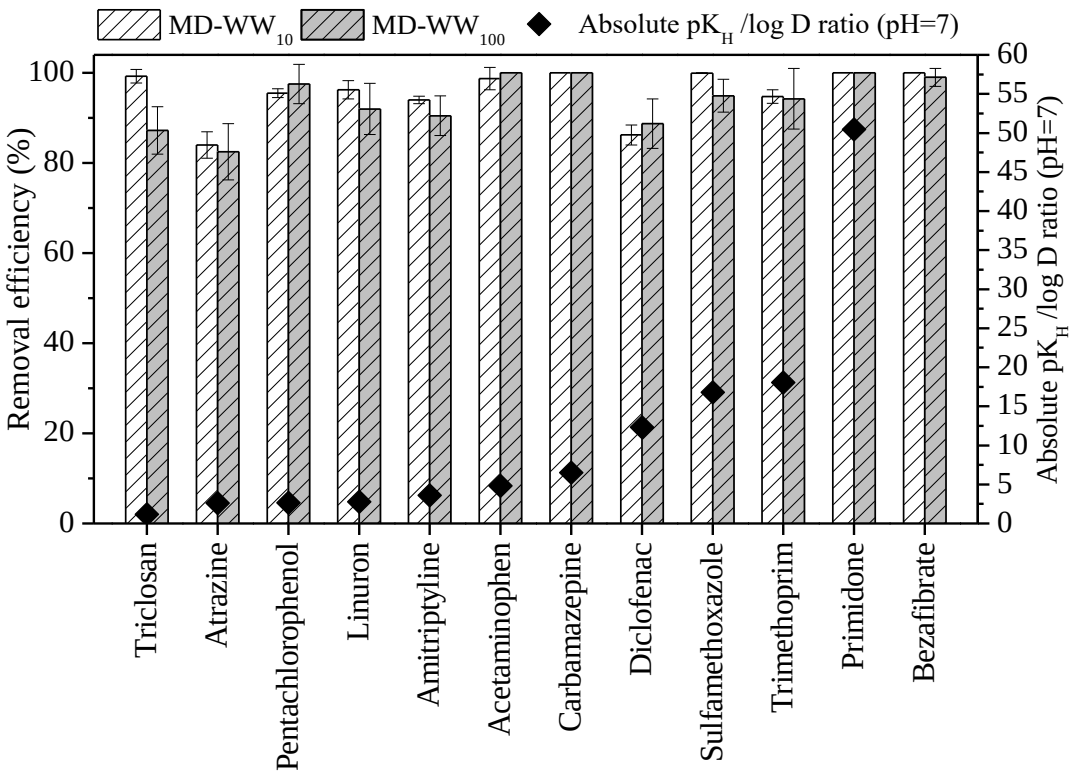

Figure 7.4. Performance of the stand-alone MD for the removal of the selected TrOCs arranged based on pK_H /log in presence of metal salts at 10 (MD-WW_{10}) and 100 mg/L (MD-WW_{100}). Mean removal efficiency and standard deviation (n=4) are presented. Operating conditions are presented in the caption of **Figure 7.3**.

7.4.2.2. Removal of TOC, TN and metal ions

Overall removal of bulk organics was monitored *via* TOC and TN concentration in the MD permeate (distillate) for different wastewater compositions (**Table 7.4**). TN and TOC removal by the stand-alone MD was consistently above 95% and 98%, respectively, as shown in **Table 7.4**, thus ensuring high quality treated effluent. However, effective retention of TOC and TN during continuous feeding also means their accumulation in MD feed tank (*i.e.*, MD reactor), which may cause severe membrane fouling [27]. This aspect is more comprehensively discussed in **Section 7.4.4**.

Table 7.4. Removal of different pollutants during the treatment of wastewater by the stand-alone MD system

Parameters	**Removal efficiency (%)**			
	Control	**MD-WW_0**	**MD-WW_{10}**	**MD-WW_{100}**
TOC	–	99	98	99
TN	–	96	98	98
Conductivity	99	99	99	99
Iron (II)	100	–	99	99
Magnesium	99	–	99	100
Calcium	99	–	100	99
Lithium	99	–	100	100

n=5 for TOC and TN, and n=4 for metal ions. Standard deviation during all analysis was below 5%. '–' indicates that metal ions were not added during that run. TrOC concentration was kept at 5 µg/L during all experiments.
The numbers in the sub-script of the run ID indicate the concentration of each metals salt. For example, MD-WW_0 represents the run with no metal salts, while MD-WW_{10} indicates that the concentration of each salt during this experiment was 10 mg/L.

Composition of AMD-impacted water is diverse and is commonly characterized by high concentrations of iron (II) as well as other trace elements such as calcium and lithium [1, 4, 5]. The MD performance was assessed for the removal of metal ions at different concentration to check process stability. According to the results presented in **Table 7.4**, removal of all metal ions was greater than 99% during all experiments. Since the water moves across the membrane in vapor form, MD can provide effective removal of non-volatile metal ions [15, 19]. However, high concentrations of metal ions can cause severe scaling, affecting membrane permeability [36] as discussed in **Section 7.4.4**.

7.4.3. Performance of an integrated MD system

7.4.3.1. Degradation of TrOCs in batch tests

To choose an oxidation process to be integrated with MD, performance and stability of both laccase and PS was assessed in batch experiments. The batch reactors (250 mL) containing the wastewater prepared by adding the mixture of TrOCs and iron salt in MBR permeate were incubated for a period of 24 h at 20 and 40 °C in separate runs. Oxidation of TrOCs by laccase is principally controlled by two factors: (i) the nature of functional groups attached to the core part of the molecule; and (ii) relative redox potential of laccase and TrOCs. Laccase can efficiently degrade phenolic compound, but the oxidation of non-phenolic compounds may be restricted by kinetic limitations [37, 38]. In this experiment, all the tested TrOCs except triclosan, pentachlorophenol and acetaminophen were non-phenolic, and their degradation by laccase ranged from 6 to 75% at 20°C, and 6 to 90% at 40°C (**Figure 7.5**). Out the 12 tested TrOCs, degradation of one non-phenolic compound (bezafibrate) and two phenolic TrOCs (triclosan, and acetaminophen) was above 75% and 90%, respectively. When the temperature of the bioreactor was increased from 20 to 40°C to assess laccase performance at the operating temperature of MD feed, degradation of three trimethoprim, diclofenac, triclosan, pentachlorophenol and acetaminophen improved significantly by 10-25%. This is because the increase in temperature can increase the rate of TrOC oxidation [39].

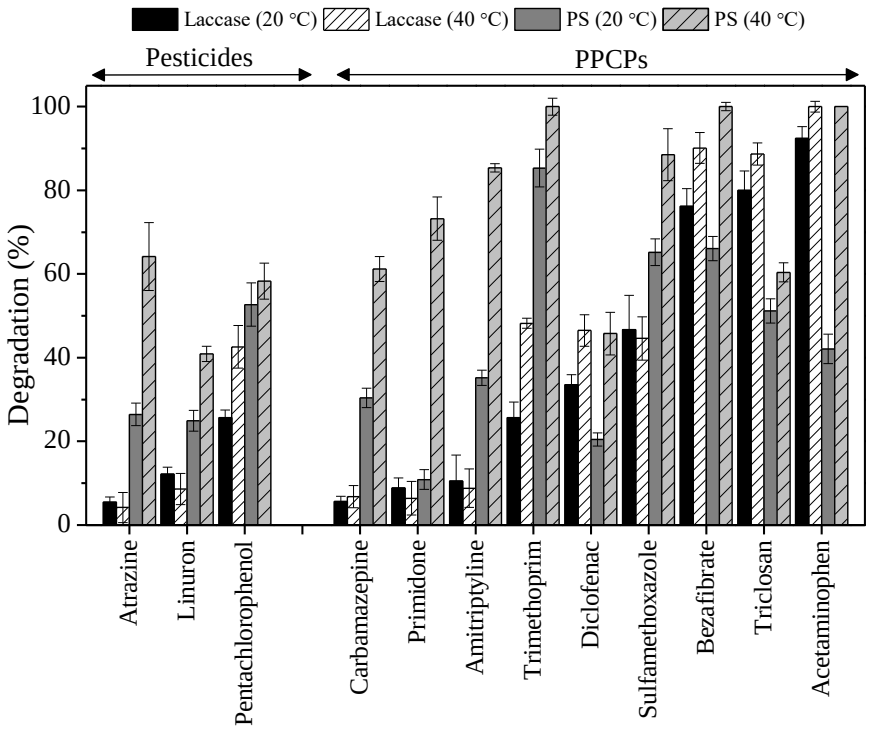

Figure 7.5. Performance of laccase and persulfate (PS) for degradation of the selected TrOCs in batch experiments. The performance of both laccase (95-100 µM/min) and PS (1 mM) was assessed for an incubation period of 24 h in presence of iron salt (10 mg/L) at 20 and 40 °C. Mean removal efficiency and standard deviation (n=2) are presented.

PS is stable at room temperature, but can be activated by various agents such as transition metals (*e.g.,* iron), heat, and ultraviolet (UV) light to form one or more sulphate radicals ($SO_4^{-\bullet}$), which are highly reactive [30]. PS activation by heat and UV light produce two $SO_4^{-\bullet}$ radicals (Equation 10), while only one $SO_4^{-\bullet}$ radical is generated following activation by transition metals such as Fe^{2+} (Equation 11). This indicates that activation by heat or UV light may provide more efficient treatment compared to activation by a transition metal [30, 40].

$$S_2O_8{}^{2-} + heat\ or\ UV\ light \rightarrow 2\,SO_4{}^{2-\cdot} \qquad \text{Eq. (10)}$$

$$S_2O_8{}^{2-} + Fe^{+2} \rightarrow Fe^{+3} + SO_4{}^{2-\cdot} \qquad \text{Eq. (11)}$$

In this experiment, ability of PS for TrOC degradation studied at 20 and 40°C to show that both heat and iron (II) can activate PS. Previously, a combined peroxymonosulfate (50 µM) – Fe^{2+} (50 µM) process achieved above 99% degradation of atrazine, outperforming atrazine removal by coagulation [41]. Heat-activated PS has been also reported to achieve 40-100% removal of a few investigated TrOCs such as atrazine, aniline, monochlorobenzene and 2,4-dichlorophenol [30]. In the current experiment, degradation of 10-85% was achieved by Fe-activated PS at 20°C (**Figure 7.5**), and the degradation of nine out of 12 TrOCs was less than 50%. This indicates that iron can cause activation because PS is stable at room temperature

of 20°C [30]. Notably, at 40°C, the spectrum of significantly degraded TrOC broadened as the degradation of 10 out of the 12 tested TrOCs was above 60% (**Figure 7.5**). Compared to laccase, performance of activated-PS was superior for eight TrOCs, while the degradation of three TrOCs (acetaminophen, diclofenac and bezafibrate) by both laccase and activated-PS was comparable. Notably, degradation of a phenolic PPCP triclosan by laccase was almost 20-30% higher than activated-PS (**Figure 7.5**).

Increasing the temperature from 20 to 40°C caused laccase inhibition. The initial laccase activity reduced by less than 5% at 20°C, while a reduction of 45% in laccase activity was observed at 40°C. On the other hand, depletion of PS was 2% at 20°C and 12% at 40°C. Thus, PS-assisted oxidation process was selected for integration with MD.

7.4.3.2. Degradation of TrOCs by PS-assisted MD system

In a stand-alone MD, TrOCs accumulate within the feed following their retention by the MD membrane. Over time, this may affect TrOC retention. This also requires additional intensive treatment of MD-concentrate that needs to be periodically purged from the system. Thus, intermittent PS dosing was investigated for TrOC degradation to reduce their accumulation in feed (with and without metal salts) during MD operation.

Following the absorption of heat, breaking of the peroxide bond (O–O) that bridges the sulphur atoms in persulfate occurs, resulting in the formation of two $SO_4^{-\bullet}$ radicals as shown in Equation 10 [30]. Depending on wastewater characteristics, persulfate or $SO_4^{-\bullet}$ radicals may react with water and/or organics to form secondary radicals that can also contribute to degradation of organic impurities [30, 42]. $SO_4^{-\bullet}$ radicals can react with water to form hydroxyl ($OH^{-\bullet}$) radicals, but the abundance of the $SO_4^{-\bullet}$ and $OH^{-\bullet}$ radicals is governed by the pH of reaction media (Equation 12 and 13). Under acidic conditions (pH<7), $SO_4^{-\bullet}$ radicals are the dominant species, while $OH^{-\bullet}$ is the primary reactive species under basic conditions (pH>7). At neutral pH, both $SO_4^{-\bullet}$ and $OH^{-\bullet}$ radicals contribute equally to pollutant degradation [43]. Since the pH of the secondary treated effluent in this experiment ranged between 6.9 and 7.2, both $SO_4^{-\bullet}$ and $OH^{-\bullet}$ radicals were responsible for the degradation of TrOCs.

$$SO_4{}^{2-\cdot} + H_2O \rightarrow SO_4{}^{2-} + OH^{\cdot} + H^+ \qquad (pH < 7) \qquad \text{Eq. (12)}$$

$$SO_4{}^{2-\cdot} + OH^- \rightarrow SO_4{}^{2-} + OH^{\cdot} \qquad (pH > 7) \qquad \text{Eq. (13)}$$

A mass balance (Equation 9) reveals that heat-activated PS achieved 25-100% degradation of the TrOCs (**Figure 7.6**) and above 99% overall removal (*i.e.,* degradation+membrane retention). During treatment without the addition of metal salts (MD-WW_0), degradation of TrOCs can be divided into three categories: (i) 90-100% degradation of four PPCPs, namely amitriptyline, trimethoprim, bezafibrate and acetaminophen; (ii) 60-90% degradation of three pesticides (atrazine, linuron and pentachlorophenol) and four PPCPs (carbamazepine, triclosan,

sulfamethoxazole and primidone); and (iii) less than 25% degradation of the pharmaceutical compound diclofenac (**Figure 7.6**). Similar to biodegradation [37, 38, 44], degradation of TrOCs by the heat-activated PS appears to be governed by their chemical structure (*e.g.*, presence of EWGs and/or EDGs). For instance, TrOCs such as amitriptyline, trimethoprim and bezafibrate that contain amine (– NH_2), alkyl (–R) or acyl (– COR) EDGs were readily degraded (**Figure 7.6**). This is because sulphate radicals are electrophilic and can achieve faster degradation of pollutants containing strong EDGs [45]. However, even some of the compounds with strong EWGs underwent significant degradation. Of particular interest was the significantly higher PS-mediated degradation of pesticides, particularly atrazine and linuron, compared to biodegradation by conventional activated sludge and fungal enzymes [37, 38, 44].

Literature on the degradation of TrOCs by heat-activated PS is scarce, and to date has been generally focused on PS activation routes in the presence of a single TrOCs. For instance, Deng et al. [46] reported only 12% degradation of carbamazepine following 2 h treatment with heat-activated-PS at a PS concentration and operating temperature of 1 mM and 40ºC, respectively. In a study by Ji et al. [47], PS (1 mM) activated by heat at 40ºC achieved 20% atrazine degradation after an incubation time of 120 h. Ji et al. [48] observed complete degradation of the antibiotic sulfamethoxazole within 8 h at 50ºC. These previous experiments were done in batch mode. Instead of a single TrOCs, this chapter presents the performance of activated PS for the degradation of a dozen of TrOCs in their mixture for the first time. Furthermore, this is the first set of data from a reactor operated in continuous-feeding mode. Although a direct comparison with previous data [46, 47] is not recommended due to the differences in experimental setup, higher degradation efficiencies observed in the current experiment are worth noting.

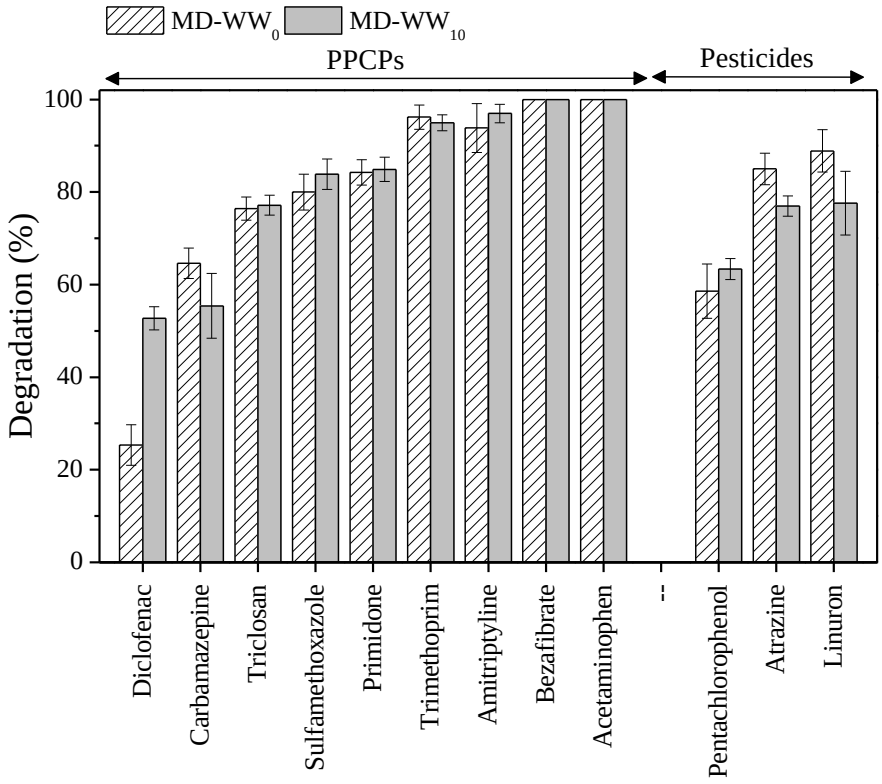

Figure 7.6. Degradation of the selected TrOCs by PS (1 mM)-assisted MD system during the treatment of wastewater with (MD-WW_{10}) and without (MD-WW_0) the addition metal salts. Data is presented as average ± standard deviation (n=4 for MD-WW_{10} and n=10 for MD-WW_0). Operating conditions are presented in the caption of **Figure 7.3**.

Notably, when metals salts each at 10 mg/L were added in MBR-permeate (MD-WW_{10}), the extent of degradation of the selected TrOC was comparable to that achieved in absence of metal salts. Importantly, degradation of a nonsteroidal anti-inflammatory drug diclofenac was increased from 25 to 52% (**Figure 7.6**). Compared to an integrated activated sludge-MD system, degradation of a few TrOCs in the PS-assisted MD system was more efficient. For instance, Wijekoon [49] reported less than 30% removal for diclofenac, atrazine and carbamazepine in an activated sludge-MD system. In the current experiment, PS-assisted MD system achieved 55-64% degradation of carbamazepine, and 76-85% degradation of atrazine (**Figure 7.6**). Future studies are recommended to systematically compare biodegradation vs. advanced oxidation-assisted MD process. However, that is beyond the scope of this chapter.

The concentration of the PS added to the MD reactor was monitored to determine if recurrent dosing of PS was required. Only a few studies have investigated the depletion of sulphate radicals during TrOC degradation [42, 50-52]. The radicals (*e.g.,* $SO_4^{-\bullet}$ and $OH^{\bullet}$) formed following PS activation by heat and/or iron (II) not only can react with the target pollutants but can also react with other radicals and non-target pollutants. The scavenging reactions (*i.e.,* radical-radical and radical-nontarget) produce secondary radicals that can take part in the degradation process. However, scavenging reactions deplete PS by converting the $SO_4^{-\bullet}$

radicals into sulphate ions [42, 45]. Depletion of PS necessitates its intermittent dosing to maintain the performance of the oxidation process. In this experiment, the concentration of persulfate was observed to be reduced by 50-70% over a period of 2×HRT. Thus, intermittent dosing of PS after every 2×HRT was applied to reinstate PS concentration to 1 mM and maintain PS-mediated degradation. Although the addition of PS would increase the operating cost of the treatment system, it is compensated generously by: (i) achieving improved TrOC removal in MD; (ii) reducing the accumulation of organic impurities in the feed of MD (See S**ection 7.4.3.3**); and (iii) significantly mitigating membrane fouling (See **section 7.4.4**).

7.4.3.3. TOC and TN degradation by PS-assisted MD system

Overall removal of bulk organics was monitored *via* TOC and TN concentration in the PS-assisted MD feed and permeate (distillate). Removal of TOC, TN and metal ions by the PS-assisted MD was consistently above 99% as shown in **Table 7.5**, thus ensuring high quality treated effluent. However, effective retention of TOC and TN during continuous feeding also means their accumulation in MD feed tank (*i.e.*, MD reactor), which may cause severe membrane fouling [27].

Table 7.5. Removal of pollutants during the treatment of wastewater by the PS-assisted MD system

Parameters	**MD-WW$_0$**		**MD-WW$_{10}$**	
	Degradation (%)	Overall removal (%)	Degradation (%)	Overall removal (%)
TOC	66±4	99±0.5	71±7	99±0.5
TN	39±2	99±1	51±3	99±0.5
Iron (II)	–	100±0	–	99±1
Magnesium	–	100±0	–	100±0
Calcium	–	99±1	–	100±0
Lithium	–	99±2	–	99±1

n=5 for TOC and TN, and n=4 for metal ions.

Persulfate and $SO_4^{-\bullet}$ radicals can directly react with organic impurities (*e.g.*, humic substances) to either degrade them or form organic radicals. The complex combination of $SO_4^{-\bullet}$ chain propagation and termination reactions govern the overall degradation of organic impurities [30, 53, 54]. In a previous study, dissolved organic carbon removal by UV-activated PS (0.6 mM) was reported to be 80% after an irradiation time of 3 h [55]. Depending on the dose of PS, Deng and Ezyske [56] achieved chemical oxygen demand and ammonia-nitrogen removal of up to 95 and 80%, respectively, from landfill leachate. Consistent with previous studies, this chapter shows significant TOC and TN removal by activated-PS.

Following effective retention by MD membrane in this chapter, up to 66-71% and 39-51% degradation of TN and TOC, respectively, was achieved by the PS-assisted MD with and without the presence of metal salts (**Table 7.5**). This significantly reduced the accumulation of these impurities in the MD reactor. The advantages of combining PS oxidation with the MD process is demonstrated for the first time in this chapter. Particularly, operating the MD system

in a continuous flow (*i.e.*, continuous feeding) mode helped demonstrate the effectiveness of PS in significantly reducing the accumulation of organics within the reactor.

7.4.4. Hydraulic performance of the stand-alone and PS-assisted MD

Permeate flux of the stand-alone MD system during treatment of different wastewater compositions was monitored continuously throughout their operation in continuous-flow mode (**Figure 7.7**). Permeate flux of the stand-alone MD reduced during all experiments and was dependent on the composition of the wastewater. During the control run (Milli-Q water, 5 µg/L TrOCs and 100 mg/L metal salts), the permeate flux reduced gradually at a rate of 1.5 L/m^2.h/d, dropping to 55% of the initial flux within 5 days (*i.e.*, 6×HRT) of operation. On the hand, addition of metal salts each at 100 mg/L and TrOCs each at 5 µg/L in MBR-permeate (i.e., MD-WW$_{100}$) significantly affected the permeability of the MD membrane by reducing the permeate flux by 76% of the initial flux (**Figure 7.7**). Based on the comparison of the permeate flux achieved by the MD membrane for different wastewater compositions, it can be concluded that the presence of metal salts affected the membrane permeability during treatment in presence of metal salts as compared to that in absence of metal salts. For instance, reduction in permeate flux was only 20% during treatment of MD-permeate without metal salts, while metal salts at 100 mg/L concentrations reduced permeate flux by 76%, respectively (**Figure 7.7**).

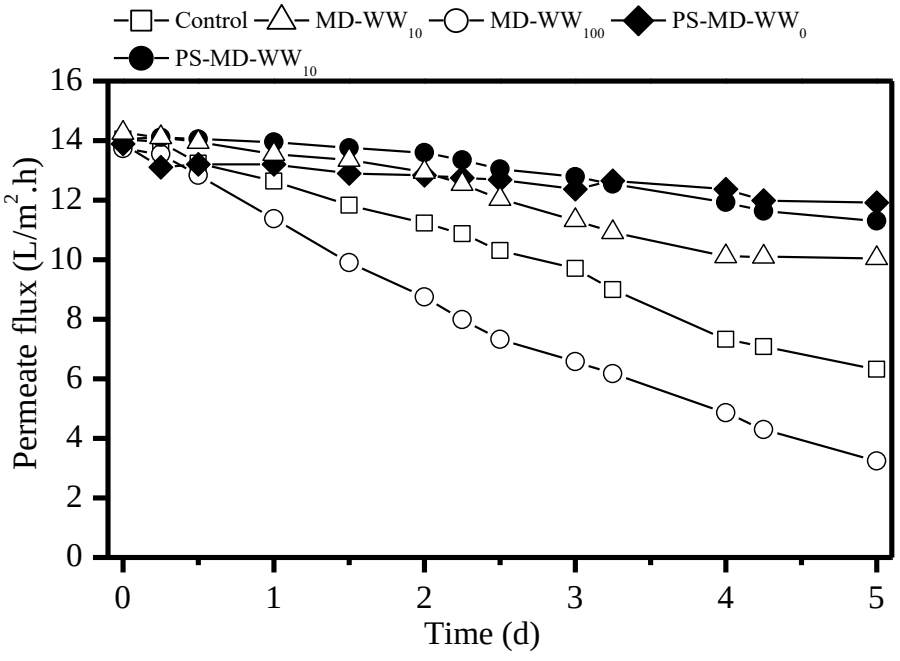

Figure 7.7. Variations in the permeate flux of the stand-alone and PS-assisted MD systems as a function of time. TrOC retention by MD during each experiment was TrOC-specific and ranged from 85 to 98% and 95 to 99% for standalone MD and integrated MD systems, respectively. Operating conditions are given in the caption of **Figure 7.3**.

Reduction in permeate flux during the stand-alone MD operation can be attributed to scaling and membrane fouling. A fouling layer formed on the membrane surface can significantly

affect permeate flux by reducing the active area of membrane surface for effective mass transfer [27, 57]. The comparatively lower flux reduction for the PS-assisted MD system can be attributed to the degradation of TOC (up to 70%, **Table 7.5**) achieved by activated PS, which reduced TOC accumulation in the feed of the PS-assisted MD system.

To derive deeper insights into the fouling phenomenon, the fouling layer formed on the membrane surface was characterized by SEM-EDS. As shown in **Figure 7.8**, during the standalone MD operation for the treatment without metal salts, a dense fouling layer formed on the membrane that almost uniformly covered the surface. On the other hand, during the PS-assisted MD operation without salt addition, the fouling layer on the membrane was distributed unevenly and covered a significantly smaller surface area (**Figure 7.8**).

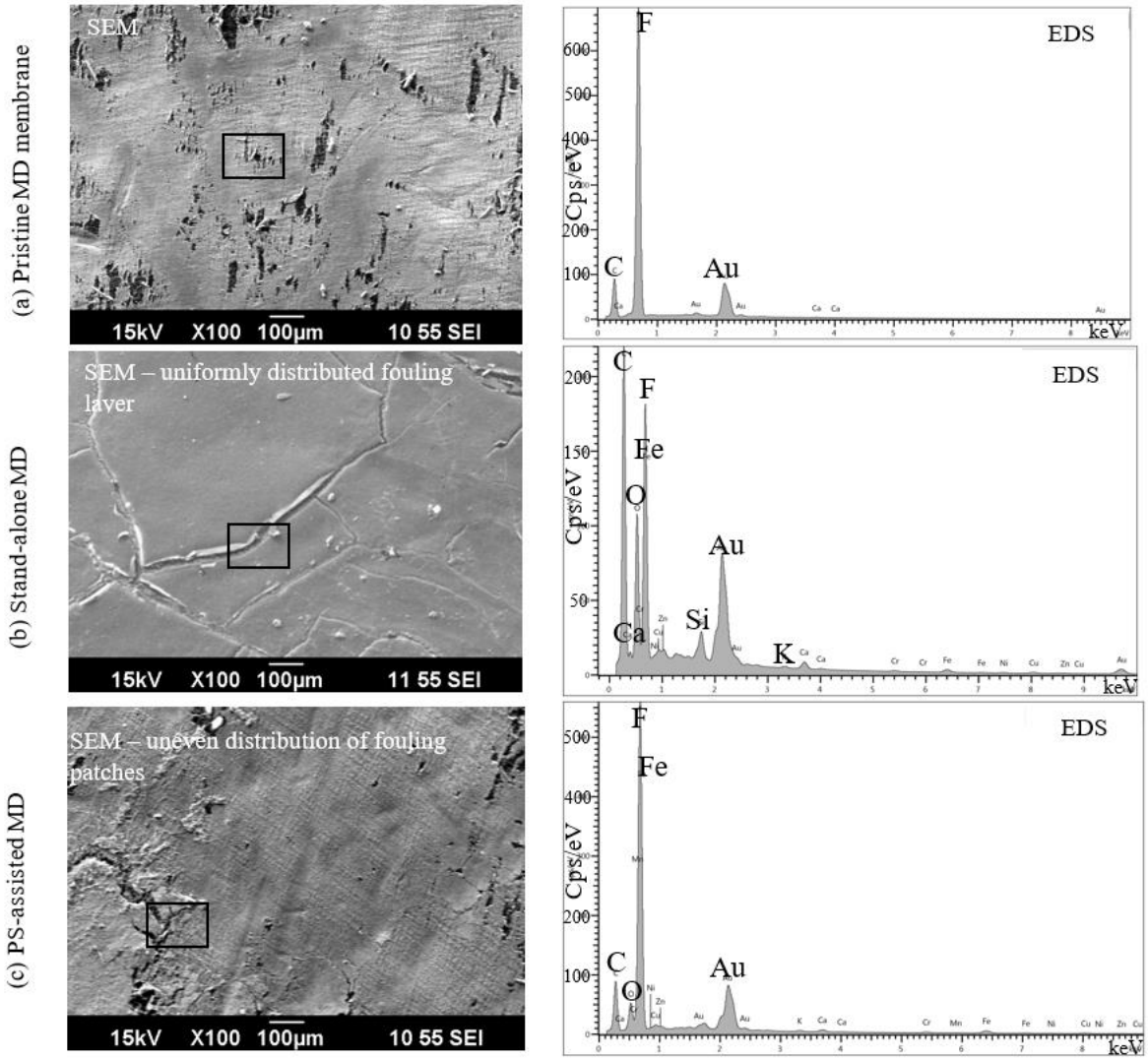

Figure 7.8. SEM images and EDS spectra of pristine MD membrane (a) and fouled membrane collected at the end of experiment with the stand-alone MD (b) and PS-assisted MD (c) systems. The membranes were used for the treatment of wastewater without the addition of metal salts.

The EDS spectra revealed that the fouling layers were mainly composed of carbon, oxygen, iron and calcium. However, the comparison of EDS spectra suggests that the abundance of carbon and oxygen (main constituents of organic impurities) was significantly higher (almost double) in the fouling layer of the membrane collected from the standalone MD system. A similar composition of fouling layer was reported when a standalone MD was operated for the treatment of anaerobic-MBR permeate [27]. Song et al. [27] additionally observed the deposition of phosphorous on the MD membrane. However, in this experiment, phosphorous was not detected in the membrane fouling layer. This can be due to the low concentration (*i.e.*, 3.4 – 6.1 mg PO_4^{3-}-P/L) of phosphorous in wastewater used in the current experiment as compared to that reported for anaerobically treated effluent in the study by Song et al. [27], *i.e.*, approximately 200 mg PO_4^{3-}-P/L.

MD membrane flux reduction can be also caused by accumulation of salts leading to concentration polarization and membrane scaling [17]. However, in this experiment, at the end of the operation, the conductivity of the feed increased from 200 to 2050 µS/cm in case of the standalone MD system in absence of additional metal salts, which is comparable to the increase observed for the PS-assisted MD system (*i.e.*, from 190 to 2940 µS/cm), and did not affect permeate flux during MD-WW_0 and PS-MD-WW_0 run. However, when metal salts were added at different concentrations, the composition of fouling layer completely changed (**Figure 7.9**). The EDS spectra revealed that the fouling layers were mainly composed of carbon, oxygen, iron and calcium, but the percentage of iron is significantly higher than other components of the fouling layer. It is evident that under the operating conditions of this experiment, salt accumulation affected the permeate flux when additional metal salts were added to the MD feed. Notably, membrane pore wetting phenomenon did not occur for any of the membranes, which is evident from the effective conductivity removal (above 99%) by MD membrane in all experiments.

It is noteworthy that the fouling layer on the membrane could potentially influence the degree of removal of dissolved constituents including TrOCs. For example, for nanofiltration, membrane fouling may cause different changes in hydrophobicity, surface charge, and effective pore size of the membrane, which may lead to reduced rejection depending on the membrane evaluated and the charge of the compound [58]. Also, in the presence of a fouling layer, polymeric forward osmosis membranes may swell due to elevated electroneutrality, reducing rejection of hydrophilic non-ionic TrOCs [59]. However, results shows that despite significant fouling, removal of TrOCs, TOC, TN and metal ions by the MD membrane was stable throughout the operation period (**Figure7.3 and Table 7.4 and 7.5**).

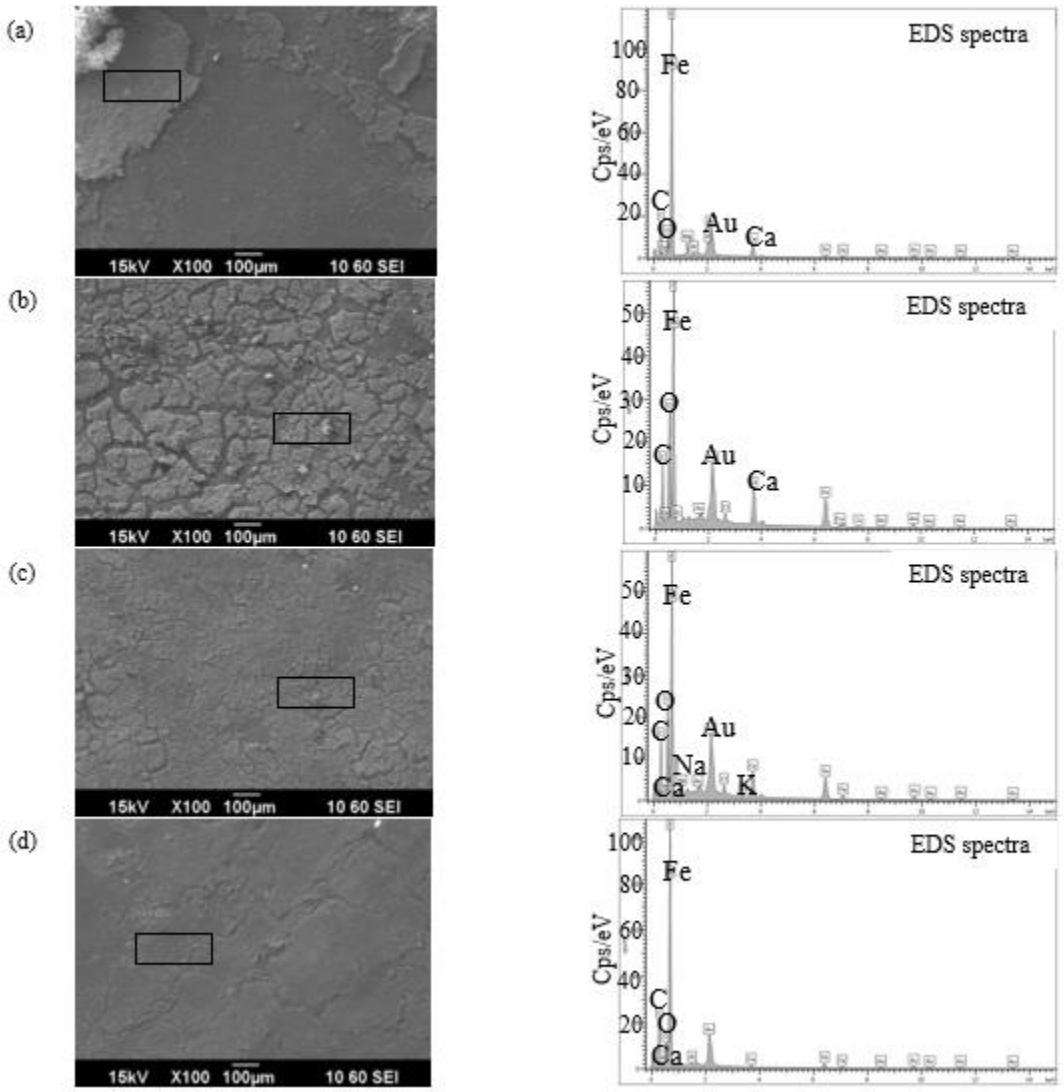

Figure 7.9. SEM images and EDS spectra of the MD membrane. (a) PS-assisted MD membrane at the end of MD-WW_{10} run; (b) MD membrane at the end of MD-WW_{10} run (c) MD membrane at the end of MD-WW_{100} run; and (d) MD membrane at the end of control run.

7.4.5. Toxicity of treated effluent

The bioluminescent bacteria *Photobacterium leiognathi* was used to monitor effluent toxicity. Our analysis indicates that the reactor media toxicity for both the stand-alone and PS-assisted MD slightly increased at the end of their operation (**Table 7.6**), and that the toxicity of the PS-assisted MD reactor media in absence of metal salts (6.3-6.5 rTU, n=2) was higher than that of the stand-alone MD (3.4-3.9 rTU, n=2). This suggests that PS itself and/or the transformation products originating from PS-mediated degradation of the organics present in the feed (*i.e.*, effluent organic matter and spiked TrOCs) was slightly more toxic than the feed. Whatever those compounds (whether PS, or intermediate TrOC transformation products) were, they did not pass into the permeate, and the MD permeate (*i.e.*, the final effluent) was not toxic to bacteria (below the assay limit of detection, <1 rTU) (**Table 7.6**).

Table 7.6. Toxicity, expressed as relative toxic unit (rTU), of different samples. The limit of detection of the toxicity assay was 1 rTU. Number of samples, n = 2.

Sample description	Toxicity (rTU)
MD feed (*i.e.*, Secondary treated effluent + TrOCs)	<1 – 2.4
Reactor media of the stand-alone MD system	3.4 – 3.9
Reactor media of the PS-assisted MD system	6.3 – 6.5
MD permeate	<1

7.5. Conclusions

This chapter aims to elucidate the performance of a direct-contact MD process for simultaneous removal of metals and TrOCs from sewage- and AMD-impacted water. The results in this chapter indicate that the stand-alone MD can achieve effective retention of both TrOCs (80-100%) and metal ions (>99%). Notably, addition of metal salts did not affect the removal of TrOCs and metal ions but caused significant fouling due to the accumulation of organic and inorganic impurities in the MD reactor. To reduce the accumulation of bulk organics and TrOCs, performance of laccase and persulfate (PS) was assessed. Based on the results of this chapter, PS exhibited better TrOC removal and stability in batch experiments as compared to laccase. Thus, PS-assisted oxidation process was selected for integration with MD. Depending on the molecular structure and hydrophobicity of the TrOCs, PS dosing at a concentration of 1 mM achieved 25 to >99% TrOC degradation with and without the addition of metal salts. This led to the consistent removal of above 99% for all the TrOCs from the MD permeate (*i.e.*, final effluent), without the production of toxic transformation products in the MD permeate. Activated PS degraded other organic impurities, along with TrOCs present in MD feed. Accordingly, during continuous operation of the PS-assisted MD system, organics accumulation in the reactor media was significantly reduced. This in turn helped minimize membrane fouling to some extent. However, when metal salts were added, performance of MD membrane was still affected due to the formation of scaling layer.

7.6. References

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Chapter 8: Conclusions and recommendations for future work

8.1. Conclusions

This thesis systematically investigates the performance of a novel high retention (HR) – enzymatic membrane bioreactor (EMBR) for effective degradation of a broad spectrum of trace organic contaminants (TrOCs) ubiquitously detected either in sewage-impacted water or in wastewater treatment plant effluent. In nutshell, two different configurations of HR-EMBRs *viz* nanofiltration (NF)-EMBR and membrane distillation (MD)-EMBR are developed and explored for the first time. The impacts of (i) laccase source; and (ii) redox-mediator (low molecular weight chemicals added to improve laccase-catalyzed degradation) type and concentration on membrane retention, TrOC degradation and effluent ecotoxicity are systematically analyzed and elucidated. In addition to redox-mediator dosing, a combined laccase/persulfate (PS)-assisted oxidation process is envisaged, and its efficacy for TrOC degradation as well as estrogenicity and ecotoxicity reduction is assessed for understanding the stability of the developed process. Simultaneous removal of both TrOCs and the metal ions by a high retention MD process is comprehensively studied by assessing the effects of organic and inorganic impurities on membrane retention and fouling.

In *Chapter 3*, integration of an enzymatic bioreactor (3 L) with a high retention NF membrane (0.2 kDa), which will retain both laccase and TrOCs, and a conventional UF membrane (30 kDa), which will only retain laccase but not TrOCs, was investigated to assess the fate of a diverse set of 29 TrOCs. The operation of both EMBRs under full recirculation mode confirmed effective retention (95% for UF membrane and 100% for NF membrane) of a commercially available laccase from genetically modified *Aspergillus oryzae*. However, during the operation of EMBRs, laccase activity may diminish due to various physical, chemical and biological inhibitors such as shear stress caused by membrane filtration. The laccase activity was maintained by re-injecting a small dose of laccase (250 µL per litre of bioreactor media). The continuous-flow NF-EMBR was observed to produce high quality effluent due to effective TrOC retention (90-99%). NF-EMBR achieved TrOC-specific improvement in the extent of degradation (up to 65%) as compared to that achieved by UF-EMBR. It is noteworthy that membrane retention and laccase-catalyzed degradation were the removal mechanism, and their contribution was dependent on TrOC properties. For instance, laccase-catalyzed degradation was the main mechanism of removal for TrOCs containing strong electron donating functional groups (EDGs), while TrOCs containing strong electron withdrawing functional groups (EWGs) were mainly removed *via* membrane retention mechanism. Although degradation is the only expected mechanism of removal in UF-EMBR, anionic and hydrophobic (log D>3) TrOCs were partially retained by the UF membrane *via* charge repulsion by UF membrane and adsorption on the enzyme gel-layer visible to the naked

eye. Change in membrane properties due to laccase adsorption along with concentration polarization reduced the permeate flux of the UF and NF membrane, but the flux can be recovered effectively by cleaning the membrane with water.

In *Chapter 4*, MD, which is another format of the high retention membrane separation process with a completely different working principle as compared to the pressure-driven NF, was integrated with an enzymatic bioreactor. Degradation of TrOCs by two commercially laccases from: (i) genetically modified *Aspergillus oryzae*; and (ii) *Trametes versicolor* was examined and elucidated. The MD system ensured complete retention (>99%) of both laccases and selected TrOCs. Laccase from *T. versicolor* and *A. oryzae* achieved 40-80% and 45-99% TrOC degradation, respectively. Laccase from *A. oryzae* demonstrated better overall performance possibly due to its higher redox-potential (up to 15%). Notably, during MD-EMBR operation, degradation of TrOCs was achieved within first 9 h regardless of laccase type. Cease of TrOC oxidation at the later end was apparently due to laccase inactivation. Hydraulic performance of MD-EMBR monitored by recording the permeate flux was stable during all experiments. Importantly, like NF-EMBR, the complete retention of the TrOCs resulted in improved TrOC degradation by both laccases as compared to previously developed UF-EMBRs. In addition to the prolonged contact time ensured by the MD process, TrOCs containing phenolic moiety such as oxybenzone and bisphenol A can improve laccase-catalyzed degradation by acting as redox-mediators. The secondary radicals or coupling agents, which are formed following the oxidation of phenolic TrOCs, are highly reactive and could directly oxidize or polymerize other TrOCs.

Chapter 5 elucidates the factors governing the performance of redox-mediators following their addition in the enzymatic bioreactor of MD-EMBR. Two N=OH type redox-mediators, namely 1-hydroxybenzotriazole (HBT) and violuric acid (VA), and one phenolic redox-mediator, namely syringaldehyde (SA), were assessed at different concentrations. The selected redox-mediators can improve TrOC degradation by following a hydrogen atom transfer pathway. Degradation of phenolic compounds and non-phenolic compounds by the MD-EMBR was improved by 20–30% and 10–50%, respectively, following the addition of redox-mediators. Improved TrOC degradation after the addition of redox-mediators is possibly due to the generation of aminoxyl and phenoxyl radicals that have significantly higher redox-potential than that of laccase only. Since SA and VA showed different patterns of TrOC-specific degradation-improvement during MD-EMBR, it was envisaged that a mediator-mixture would have further beneficial effects. Instead of inducing a synergistic effect, degradation of at least six pharmaceutically active TrOCs reduced in presence of SA-VA mixture. This is because, in mixtures, some mediators can: (i) chemically interact with each other instead of acting as an electron shuttle for laccase; and/or (ii) reversibly inhibit laccase, thereby inhibiting electron transfer between laccase and TrOCs. Indeed, laccase activity reduced by up to 80% in laccase-mediator system, indicating frequent replenishment of laccase. Mediator addition increased the

toxicity of the reaction media, but MD permeate remained non-toxic. This is an added advantage of coupling an enzymatic bioreactor with a high retention MD membrane.

Chapter 6 details the performance of an integrated laccase- and persulfate (PS)- assisted oxidation process for the first time. To date, only redox-mediators have been assessed to improve the extent of laccase-catalyzed degradation. However, they achieve TrOC degradation at the expense of laccase activity and could increase the toxicity of the treated effluent. Thus, PS-assisted oxidation process was studied as an alternative to the redox-mediators. A series of batch tests were performed at different PS concentrations ranging from 1 to 10 mM to investigate the impact of PS concentration and incubation time on TrOC degradation. Among the tested PS concentrations (1-10 mM), the best performance was achieved at 5 mM concentration. PS at 5 mM achieved 100% degradation for two phenolics (bisphenol A and oxybenzone) and 25-53% degradation for three non-phenolic (diclofenac, sulfamethoxazole, and carbamazepine) TrOCs. A continuous treatment system developed by integrating a nanofiltration (NF) membrane with laccase/PS process was hydraulically stable and achieved steady-state degradation within 24 h for all TrOCs. Importantly, degradation of non-phenolics further improved by 10 to 65% in laccase/PS-NFBR system as compared to laccase only. This could be attributed to the prolonged contact time between laccase/PS and TrOCs; as well as the contribution of oxidative coupling agents in degradation. The NF membrane not only retained the moderately degraded carbamazepine and sulfamethoxazole effectively but was also effective for the removal of the toxic transformation by-products and residual estrogenic activity as evaluated by ecotoxicity and estrogenicity assays, respectively.

Chapter 7 reports the performance of a direct-contact MD process for simultaneous removal of metal ions and TrOCs from sewage- and AMD-impacted water for a period of 6×HRT (*i.e.*, 5 d). For this purpose, different compositions of wastewater was prepared by adding a mixture of structurally diverse TrOCs as well as a mixture of four metal ions (iron, calcium, magnesium and lithium each at 10 and 100 mg/L concentration). The stand-alone MD process achieved 80-100% and >99% removal (*via* membrane retention) for the selected TrOCs and the metals, respectively. Based on the performance achieved by the MD membrane, addition of salts did not affect the extent of pollutant removal. Effective retention of bulk organics, TrOCs and metal ions during continuous feeding also means their accumulation in MD feed tank (*i.e.*, MD reactor). This caused severe membrane fouling as evident from flux reduction. Accumulation of bulk organics could be reduced by combining a laccase or activated-PS oxidation process with the MD system. The integration of PS-assisted oxidation process reduced the accumulation of bulk organics and TrOCs, but a gradual decline in permeate flux was still observed due to membrane scaling mainly caused by iron. Nevertheless, the MD membrane effectively retained all impurities, and consistently produced a high-quality effluent during all experiments.

Two different configurations of HR-EMBR are assessed for enhanced degradation of TrOCs in this thesis. Both NF- and MD-EMBRs achieved TrOC-specific improvement in degradation by laccase, but it is not appropriate to compare their performances due to difference in their working principles (temperature gradient-driven vs. pressure-driven) and operating conditions such as temperature and operating mode (concentration mode in MD and continuous-flow mode in NF). Both EMBR configurations have its own pros and cons. For instance, MD-EMBR achieved better extent of TrOC degradation, while laccase showed significantly better stability in NF-EMBR.

8.2. Recommendations for future research

This thesis provides an in-depth understanding of the TrOC removal in HR-EMBRs by elucidating the performance of laccase-catalyzed degradation, role of membrane removal mechanism, synergistic impacts of the combined laccase/mediators- and laccase/PS systems. However, few new research questions emerged during the course of this research and are worth exploring in future.

In this study, two different configurations of HR-EMBR, namely NF-EMBR and MD-EMBR are examined for improved degradation of TrOCs. Forward osmosis (FO) is another format of high retention membrane separation process and is recommended to be integrated with an enzymatic bioreactor. During the development and assessment of FO-EMBR performance, preliminary focus could be on the selection of a suitable draw solute that will not inhibit laccase. In addition, FO permeate is saline, and the selection of an appropriate process for the treatment saline product water will be critical.

TrOCs containing phenolic moiety can improve laccase-catalyzed degradation by acting as redox-mediators. The secondary radicals or coupling agents, which are formed following the oxidation of phenolic TrOCs, are highly reactive and could directly oxidize or polymerize other TrOCs. To date, only two TrOCs, namely bisphenol A and acetaminophen were demonstrated to act as redox-mediators. More phenolic TrOCs with the ability to facilitate degradation are required to be identified and examined in future studies.

Since intermittent replenishment is required to maintain enzymatic activity, use of crude enzymes can reduce the cost of the treatment system if renewable waste products such as agricultural residues are used for fungal growth. Because crude enzyme extract may contain a cocktail of enzymes and natural redox-mediators, their use can enhance the spectrum of significantly degradable TrOCs. The presence of unspent growth media in enzyme solution can increase organic loading in enzymatic treatment systems. Simple and robust enzyme purification processes should be developed in future studies.

For full-scale applications of HR-EMBRs for real wastewater treatment, laccase stability needs to be improved. One approach could be to use stabilizers. For instance, polyvinyl alcohol, polyethylene glycol (PEG), polythene and polysaccharide (*e.g.*, Ficoll) were able to improve

the stability of laccase during the treatment of bisphenol A. However, effluent toxicity may increase in the presence of PEG. Another option is to use encapsulation or carrier materials to improve laccase stability. In this regard, inert carrier materials may be preferred to avoid adsorption of denaturants. Efficacy of stabilizers and carriers needs to be assessed systematically in future studies in HR-EMBR.

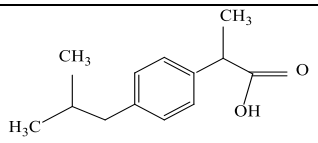
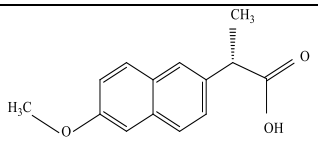
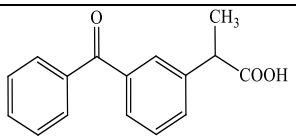
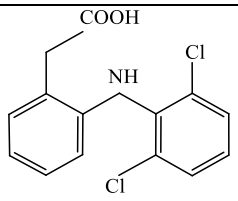
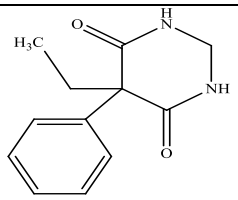
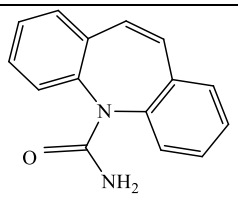
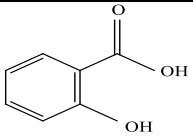
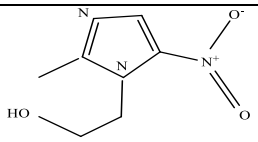
Fungal species secrete different organic compounds (*e.g.*, oxalates) that can protect them from metal-induced toxicity, their presence in the crude enzyme preparation may enhance the stability of ligninolytic enzymes. However, there is a dearth of information regarding this.

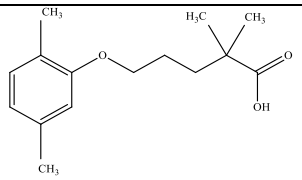
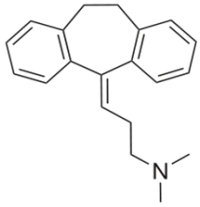
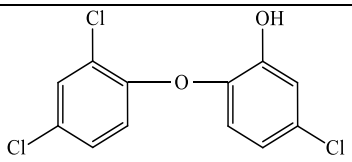
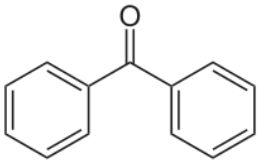
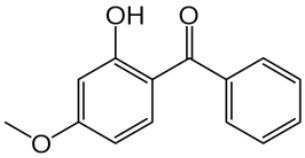
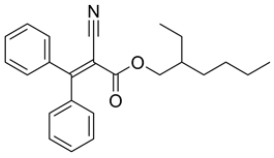
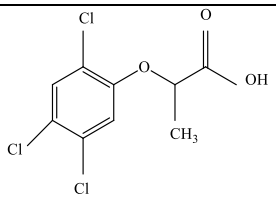
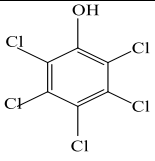
Toxicity of the reaction media, particularly following the addition of redox-mediators increase significantly, indicating that transformation by-products are more toxic than the parent compound. It is recommended to identify and elucidate the formation of transformation by-products during laccase only and/or laccase/mediator treatment process. This will explain the quantitative and qualitative difference between the transformation by-products formed in laccase-catalyzed degradation process and laccase/mediator process.

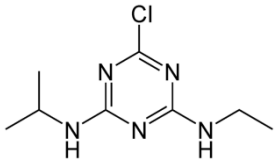
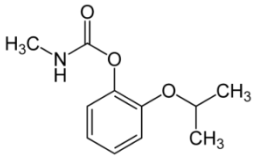
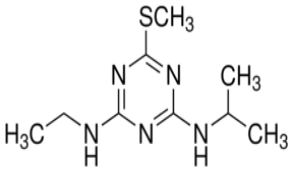
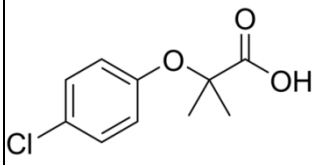
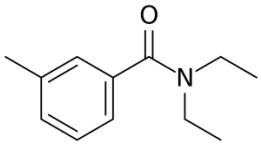
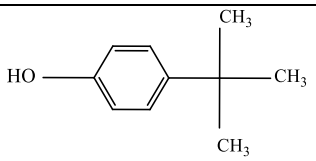
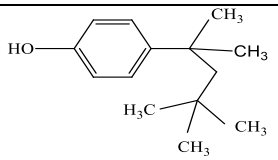
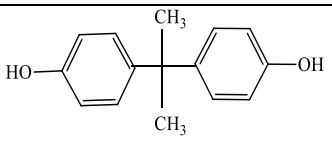
Combining laccase with a PS-assisted oxidation was observed to improve TrOC degradation without significantly inactivating laccase. Combination of other advanced oxidation processes such as photolysis and ozonation should be explored in future studies.

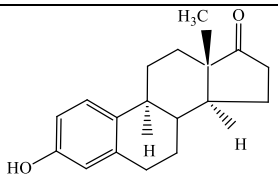
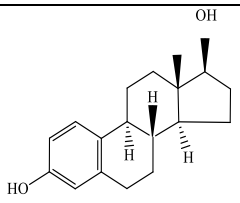
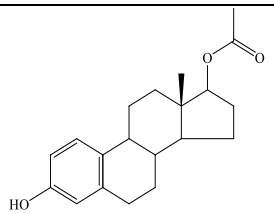
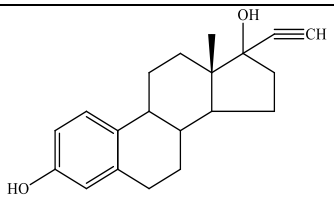
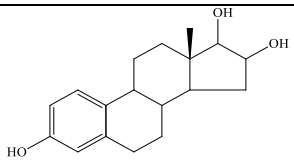
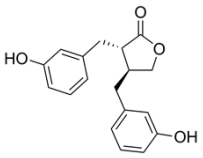
Appendix

Table 3-1. Physicochemical properties of the selected trace organic contaminants (TrOCs)

Category	Compound (Formula) (CAS number)	Molecular weight (g/mol)	Log K_{OW}[a]	Limit of detection (ng/L)[b]	Chemical structure
Pharmaceuticals	Ibuprofen ($C_{13}H_{18}O_2$) (5687-27-1)	206.28	3.50 ± 0.23	20	
	Naproxen ($C_{14}H_{14}O_3$) (22204-53-1)	230.26	2.88 ± 0.24	1	
	Ketoprofen ($C_{16}H_{14}O_3$) (22071-15-4)	254.28	2.91 ± 0.33	20	
	Diclofenac ($C_{14}H_{11}Cl_2NO_2$) (15307-86-5)	296.15	4.55 ± 0.57	5	
	Primidone ($C_{12}H_{14}N_2O_2$) (125-33-7)	218.25	0.83 ± 0.50	10	
	Carbamazepine ($C_{15}H_{12}N_2O$) (298-46-4)	236.27	1.89 ± 0.59	10	
	Salicylic acid ($C_7H_6O_3$) (69-72-7)	138.12	2.01 ± 0.25	1	
	Metronidazole ($C_6H_9N_3O_3$) (443-48-1)	171.15	-0.14 ± 0.30	20	

	Gemfibrozil ($C_{15}H_{22}O_3$) (25812-30-0)	250.33	4.30 ± 0.32	1	
	Amitriptyline C_{20} H_{23} N (50-48-6)	277.40	4.40±0.26	1	
Personal care products	Triclosan ($C_{12}H_7Cl_3O_2$) (3380-34-5)	289.54	5.34 ± 0.79	1	
	Benzophenone C_{13} $H_{10}O$ (119-61-9)	182.22	3.21 ± 0.29	5	
	Oxybenzone C_{14} H_{12} O_3 (131-57-7)	228.24	3.99±0.36	5	
	Octocrylene C_{24} H_{27} N O_2 (6197-30-4)	361.48	6.89±0.33	10	
Pesticides	Fenoprop ($C_9H_7Cl_3O_3$) (93-72-1)	269.51	3.45 ± 0.37	20	
	Pentachlorophenol (C_6HCl_5O) (87-86-5)	266.34	5.12 ± 0.36	1	

	Atrazine ($C_8H_{14}ClN_5$) (1912-24-9)	215.68	2.636±0.205	10	
	Propoxur ($C_{11}H_{15}NO_3$) (114-26-1)	209.24	1.538±0.229	1	
	Ametryn ($C_9H_{17}N_5S$) (843-12-8)	227.33	2.967± 0.12	10	
	Clofibric acid ($C_{10}H_{11}ClO_3$) (882-09-7)	214.65	2.425±0.273	1	
	DEET ($C_{12}H_{17}NO$) (134-62-3)	191.27	2.42 ± 0.23	1	
Industrial chemicals	4-tert-butylphenol ($C_{10}H_{14}O$) (98-54-4)	150.22	3.39 ± 0.21	1	
	4-tert-octylphenol ($C_{14}H_{22}O$) (140-66-9)	206.32	5.18 ± 0.20	1	
	Bisphenol A ($C_{15}H_{16}O_2$) (80-05-7)	228.29	3.64 ± 0.23	1	

Steroid hormones	Estrone ($C_{18}H_{22}O_2$) (53-16-7)	270.37	3.62 ± 0.37	5	
	17β-estradiol ($C_{18}H_{24}O_2$) (50-28-2)	272.38	4.15 ± 0.26	5	
	17β-estradiol 17–acetate ($C_{20}H_{26}O_3$) (1743-60-8)	314.42	5.11 ± 0.28	5	
	17α - ethinylestradiol ($C_{20}H_{24}O_2$) (57-63-6)	269.40	4.10 ± 0.31	10	
	Estriol (E3) ($C_{18}H_{24}O_3$) (50-27-1)	288.38	2.53 ± 0.28	10	
Phytoestrogens	Enterolactone C_{18} H_{18} O_4 (78473-71-9)	298.33	1.89± 0.37	10	

[a] Source: SciFinder database https://scifinder.cas.org/scifinder/view/scifinder/scifinderExplore.jsf
Log *D* is logarithm of the distribution coefficient which is the ratio of the sum of concentrations of all forms of the compound (ionised and unionised) in octanol and water at a given pH.

[b]Limit of detection (LOD) of the compounds during GC-MS analysis as described in Section 2.5.2. LOD is defined as the concentration of an analyte giving a signal to noise (S/N) ratio greater than 3. The limit of reporting was determined using an S/N ration of greater than 10.

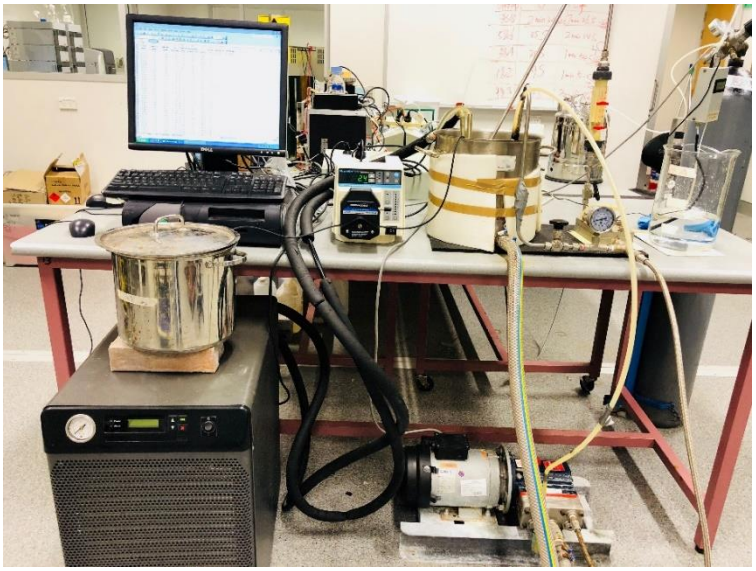


Figure 3-1. Lab-scale enzymatic membrane bioreactor

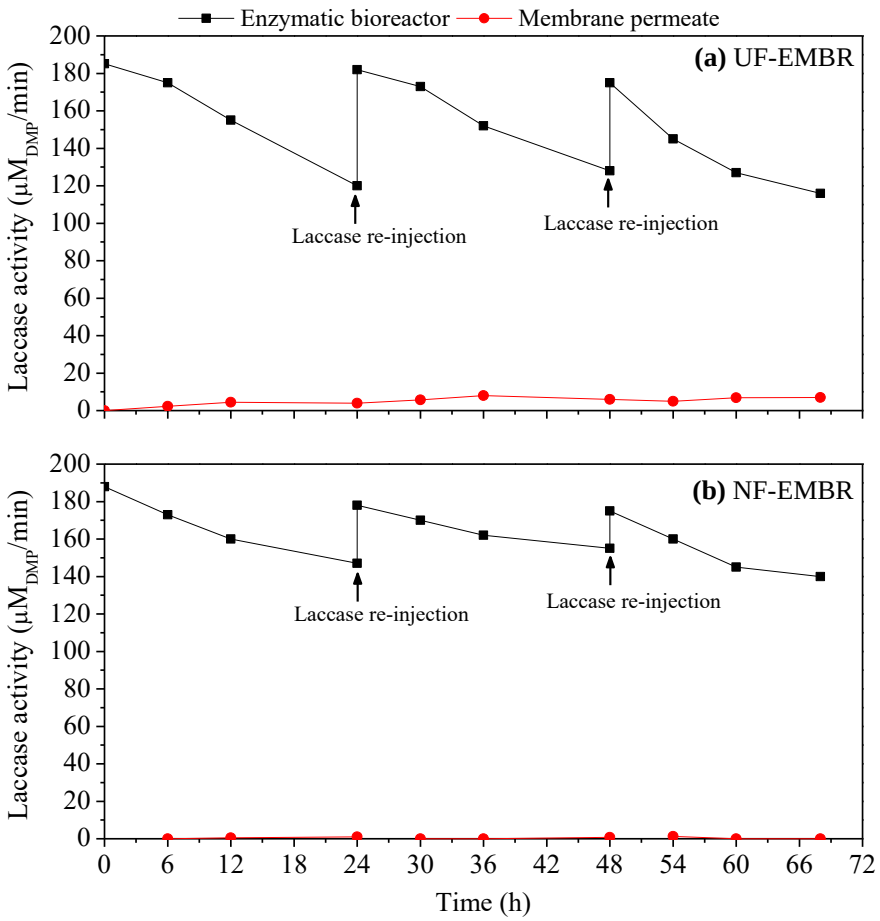


Figure 3-3. Laccase activity profiles in UF-EMBR (a) and NF-EMBR (b). Laccase activity was maintained by re-injecting a small dose of laccase (250 µL per litre of bioreactor volume) every 24 h.

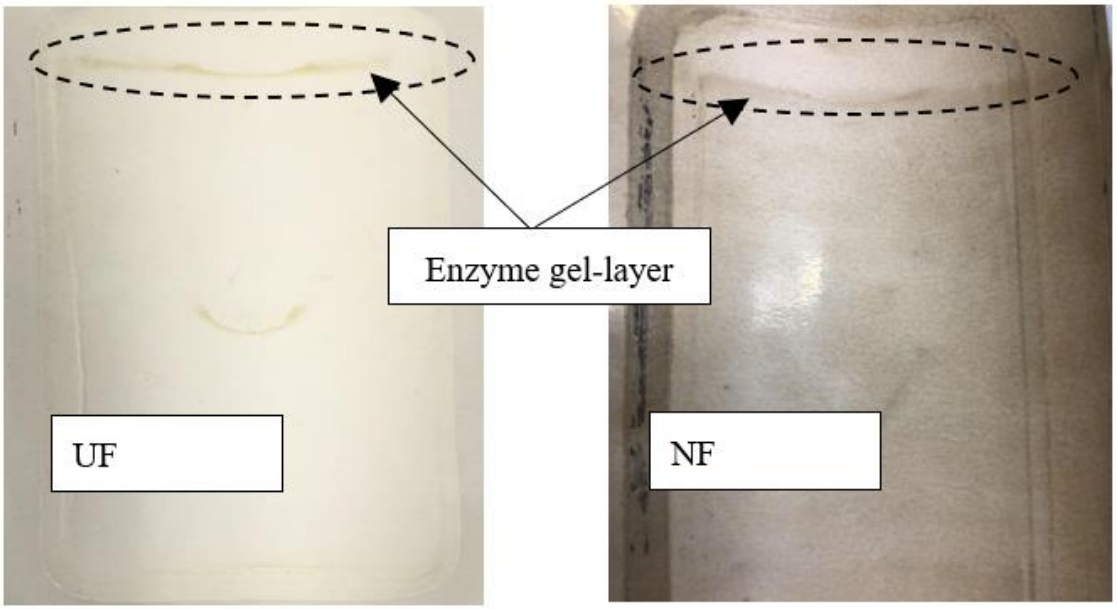


Figure 3-4. Enzyme-gel layer formed on the active side of the UF and NF membrane during the operation of UF- and NF-EMBRs.

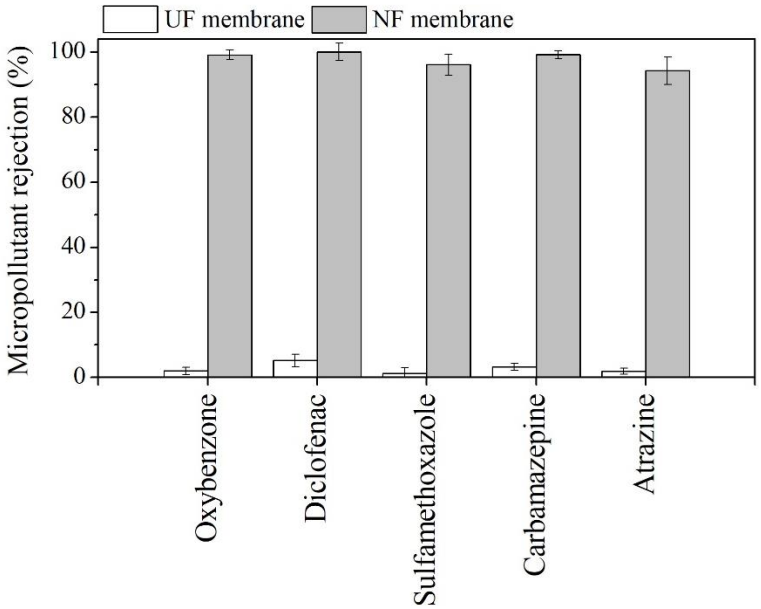


Figure 3-5. Rejection of TrOCs by the UF and NF membrane during the operation of EMBRs in full recirculation mode. Error bars show average± standard deviation (n=8).

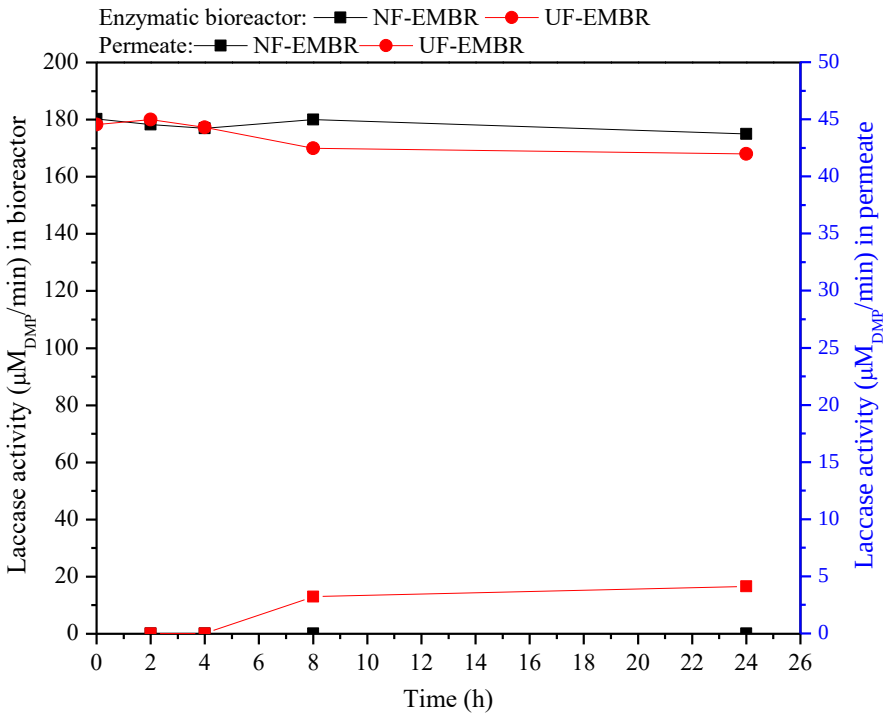


Figure 3-6. Confirmation of effective laccase retention by the NF and UF membrane. Laccase retention of >99% and 95% was achieved by the NF and UF membrane respectively. UF/NF-EMBR were operated for a period of 24 h in full recirculation mode without the addition of TrOCs.

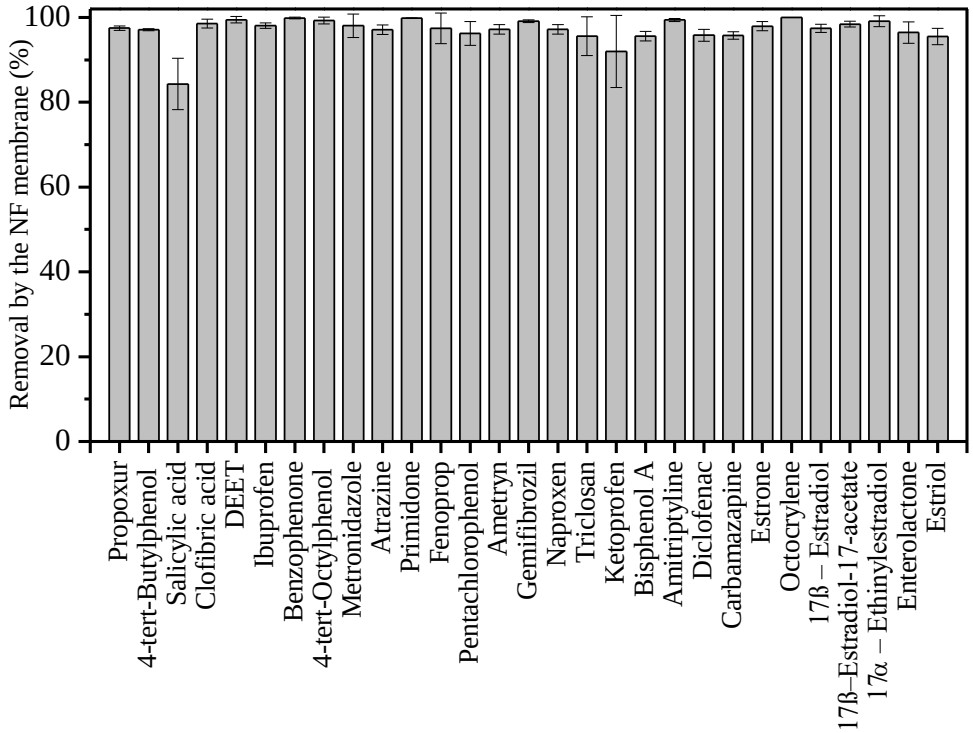


Figure 3-7. Removal of TrOC by the NF membrane to confirm their effective retention. NF-EMBR were operated for a period of 32 h in continuous mode without the addition of laccase. The UF-EMBR was not operated because the UF membrane was not expected to retain TrOCs. Data presented as average ± standard deviation (n=2).

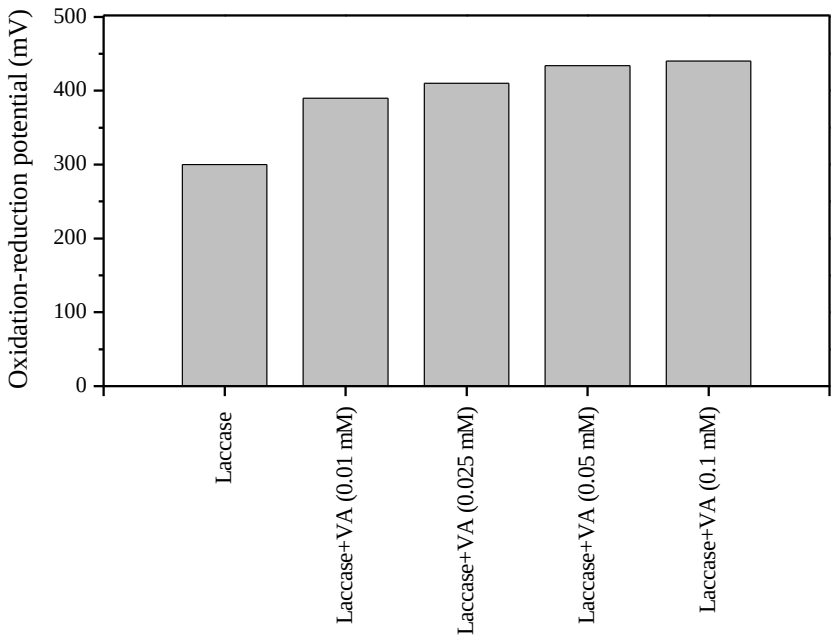


Figure 3-8. Impact of VA concentration on the oxidation reduction potential of media in NF-EMBR

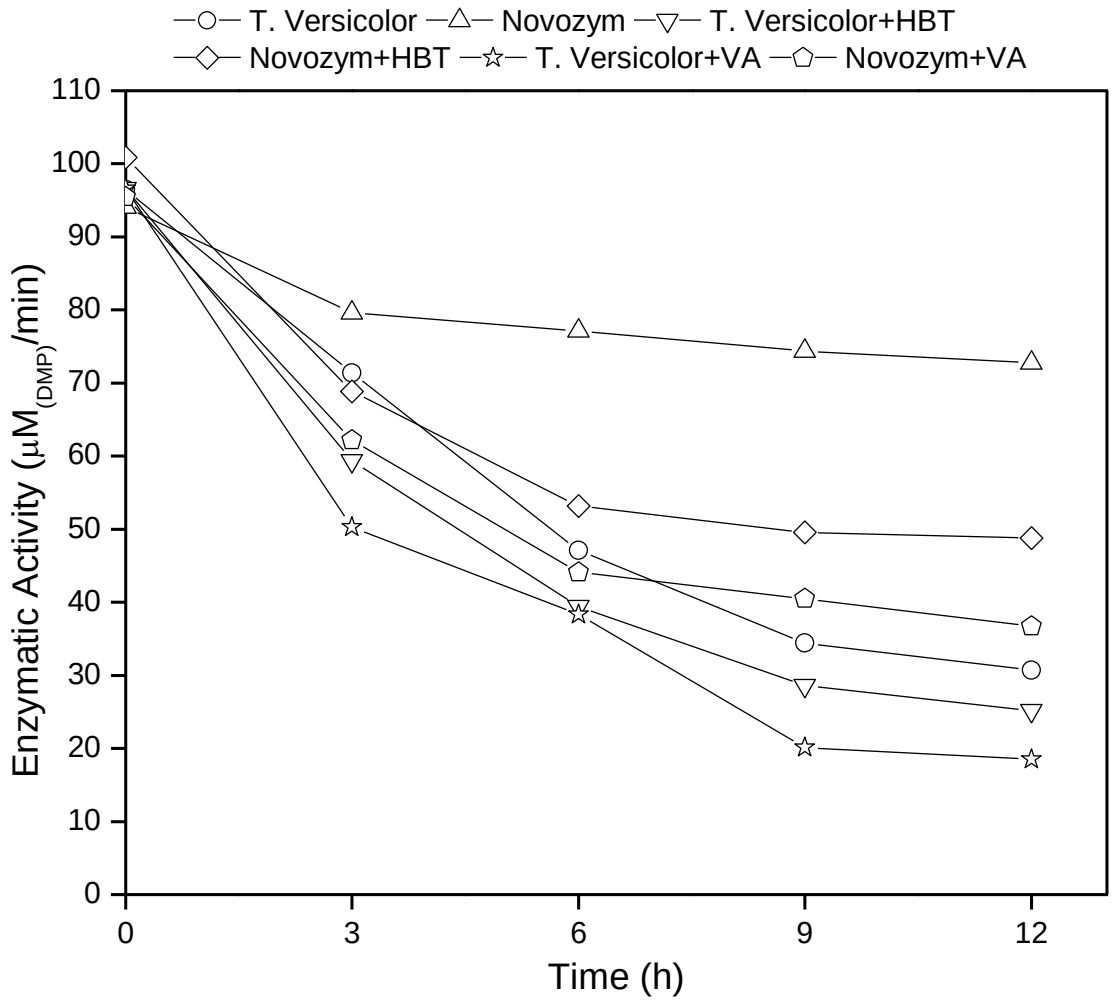


Figure 4-1. Enzymatic Activity profiles with and without the addition of redox mediator(s) in enzymatic membrane distillation system. Two N–OH type redox mediators namely, 1-hydrozybenzotriazole (HBT) and violuric acid (VA), were added separately at 1mM concentration.

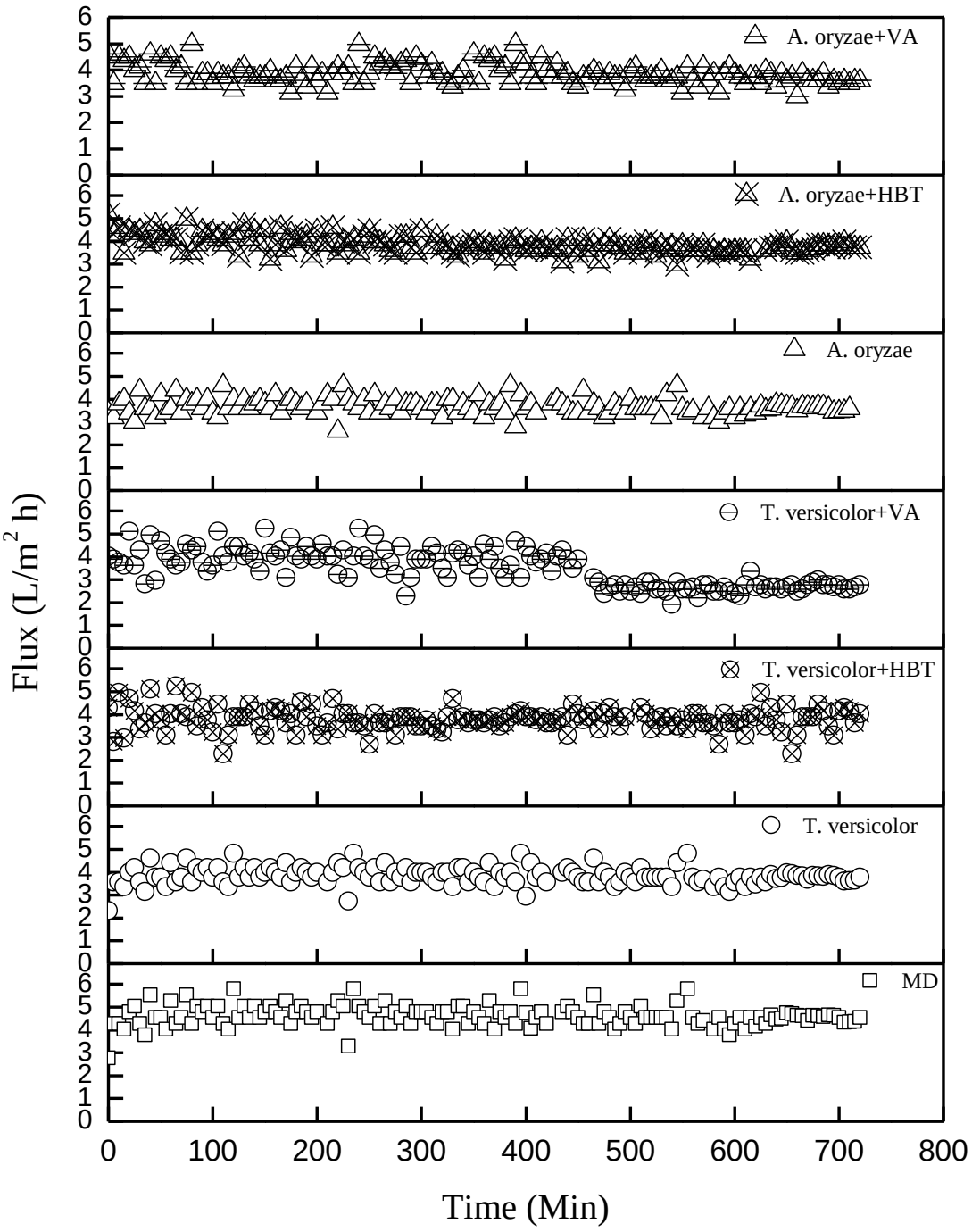


Figure 4-2. Variations in permeate flux during the operation of MD and MD-EMR systems. Feed and distillate temperature were controlled at 30 and 10 °C, respectively. The cross-flow rate of both feed and distillate side was set at 1 L/min (corresponding to a cross-flow velocity of 9 cm/s). Concentration of both HBT and VA was 1 mM.

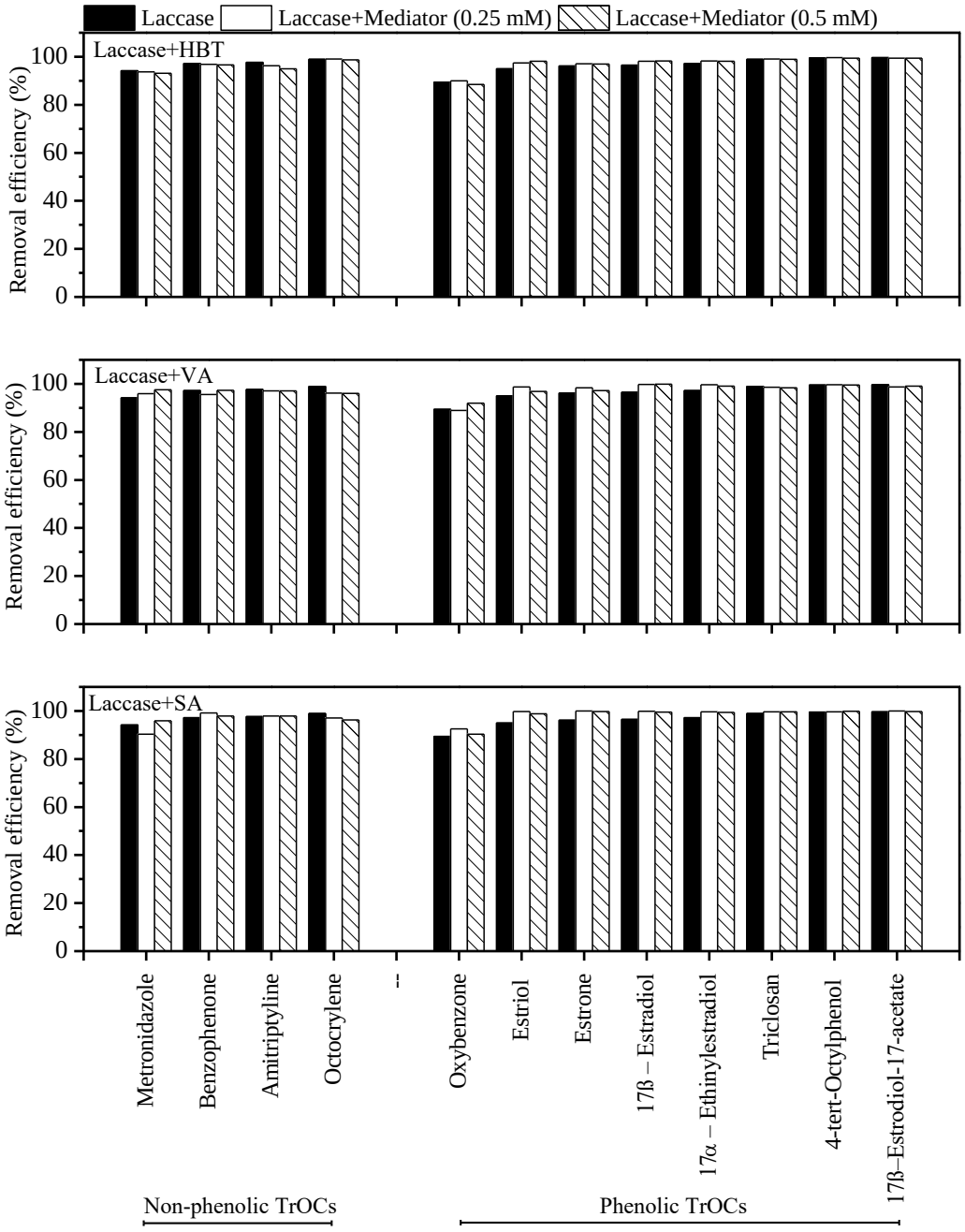


Figure 5-1. Impact of mediator concentration (0.25 and 0.5 mM) on the degradation of after an incubation time of 12 h in the MD-EMBR. Error bars indicate the standard deviation of duplicate samples. Degradation of these TrOCs did not improve by increasing mediator concentration

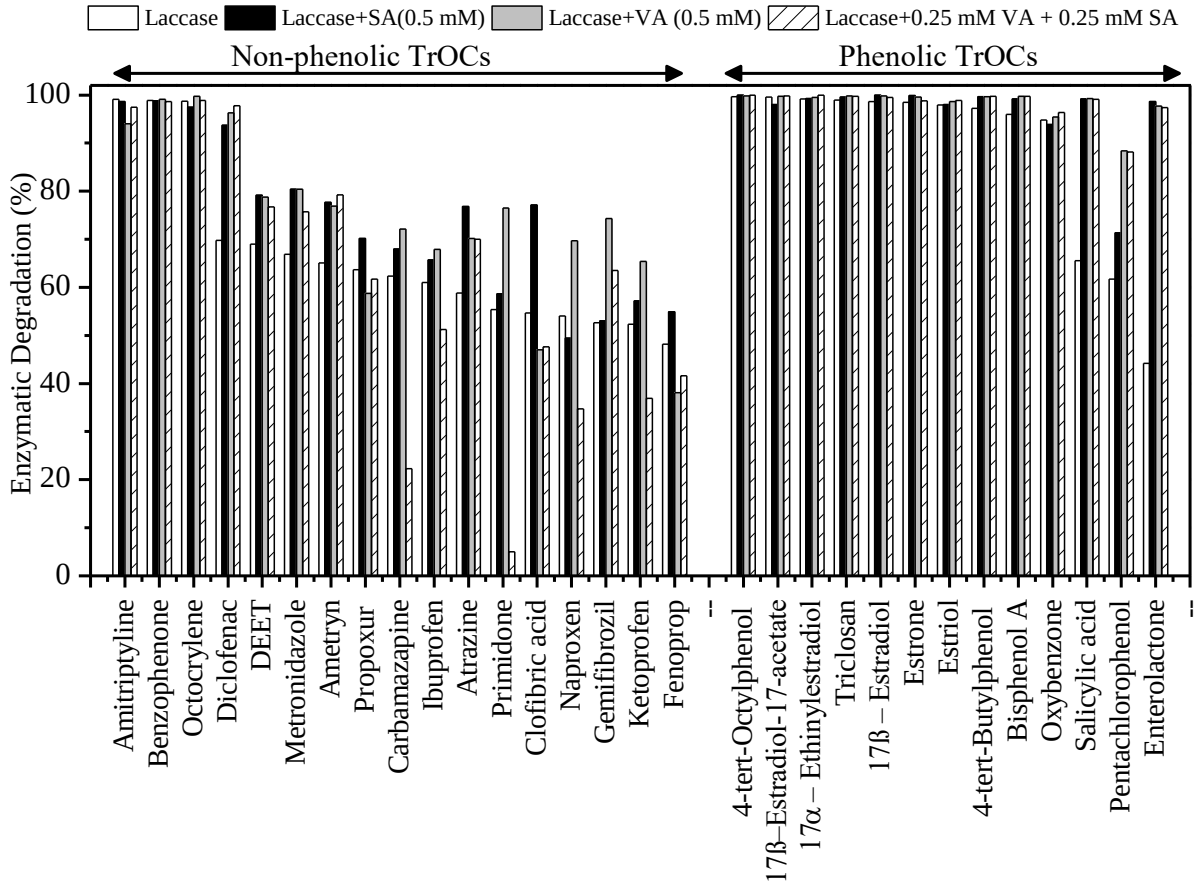


Figure 5-2. Comparison of the degradation capacity of laccase, SA, VA and SA-VA mixture in MD-EMBR operated for a period of 60 h.

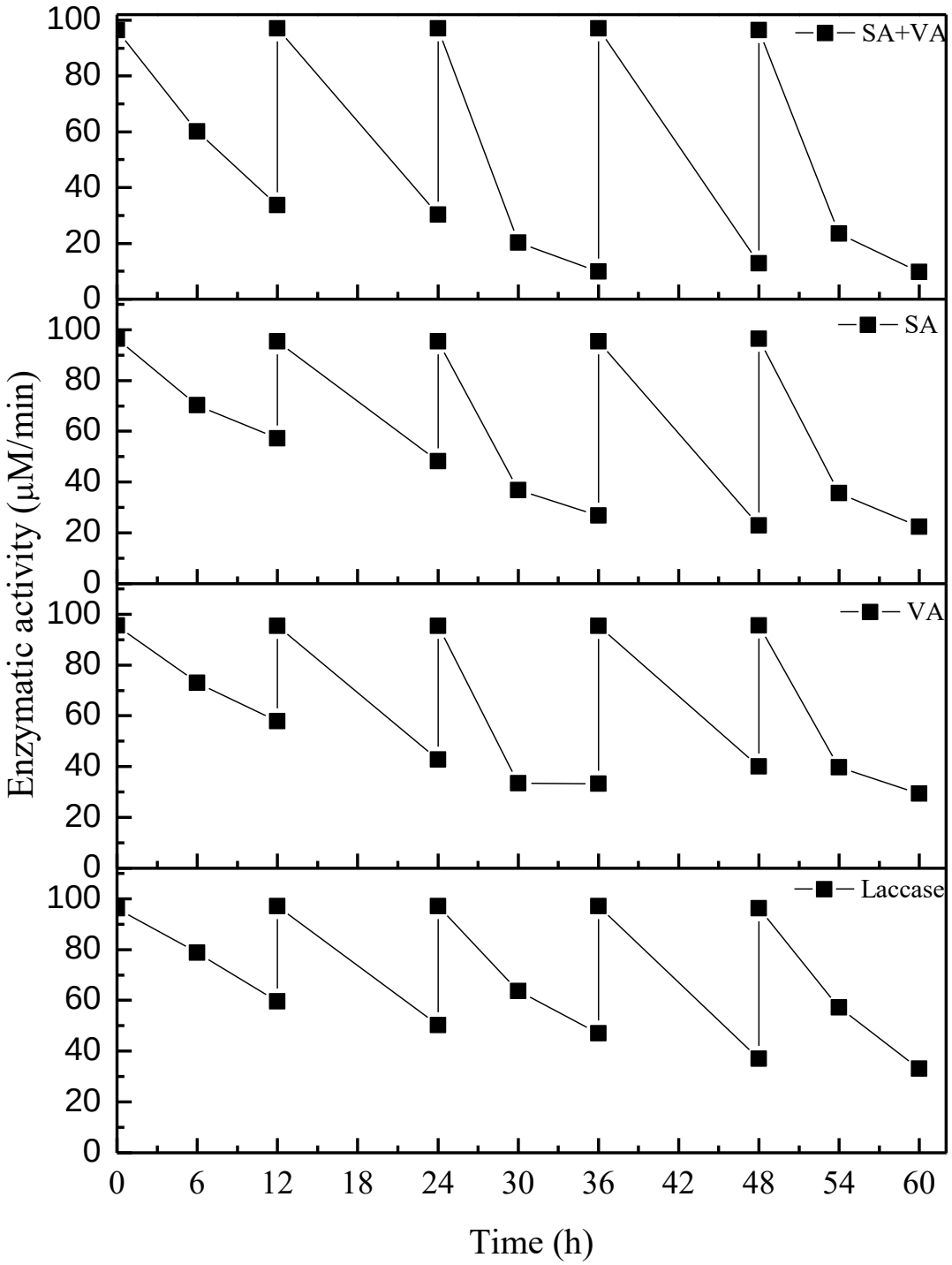


Figure 5-3. Enzymatic activity profiles with and without the addition of redox mediator(s) in EMBR during long-term operation (60 h) of MD-EMBR

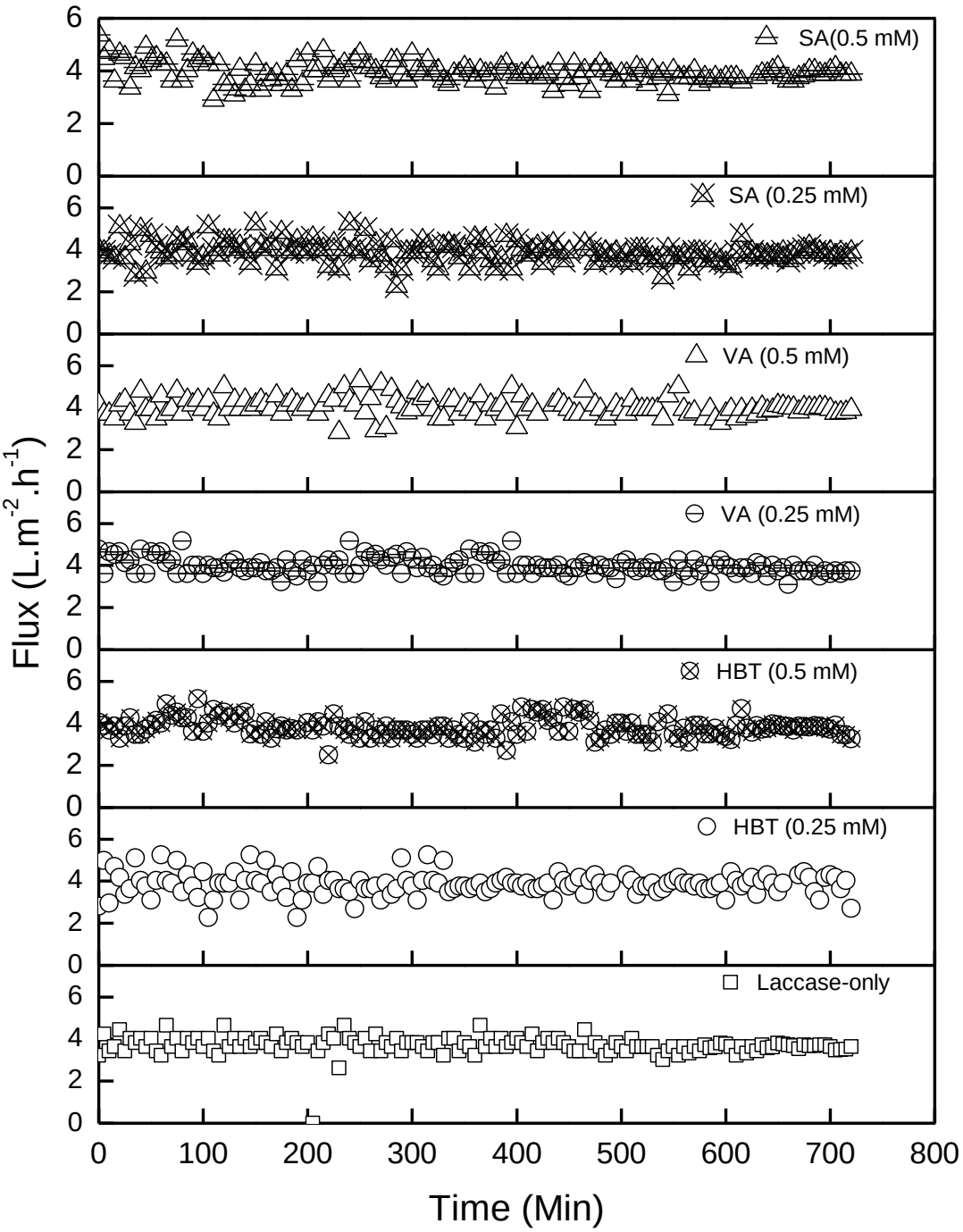


Figure 5-4. Permeate flux obtained during the preliminary operation (12 h) of enzymatic membrane distillation (MD-EMBR) with and without the addition of redox mediators. Feed and distillate temperature were controlled at 30 and 10 °C, respectively during all experiments. The cross-flow rate of both feed and distillate side was set at 1 L/min (corresponding to a cross-flow velocity of 9 cm/s).

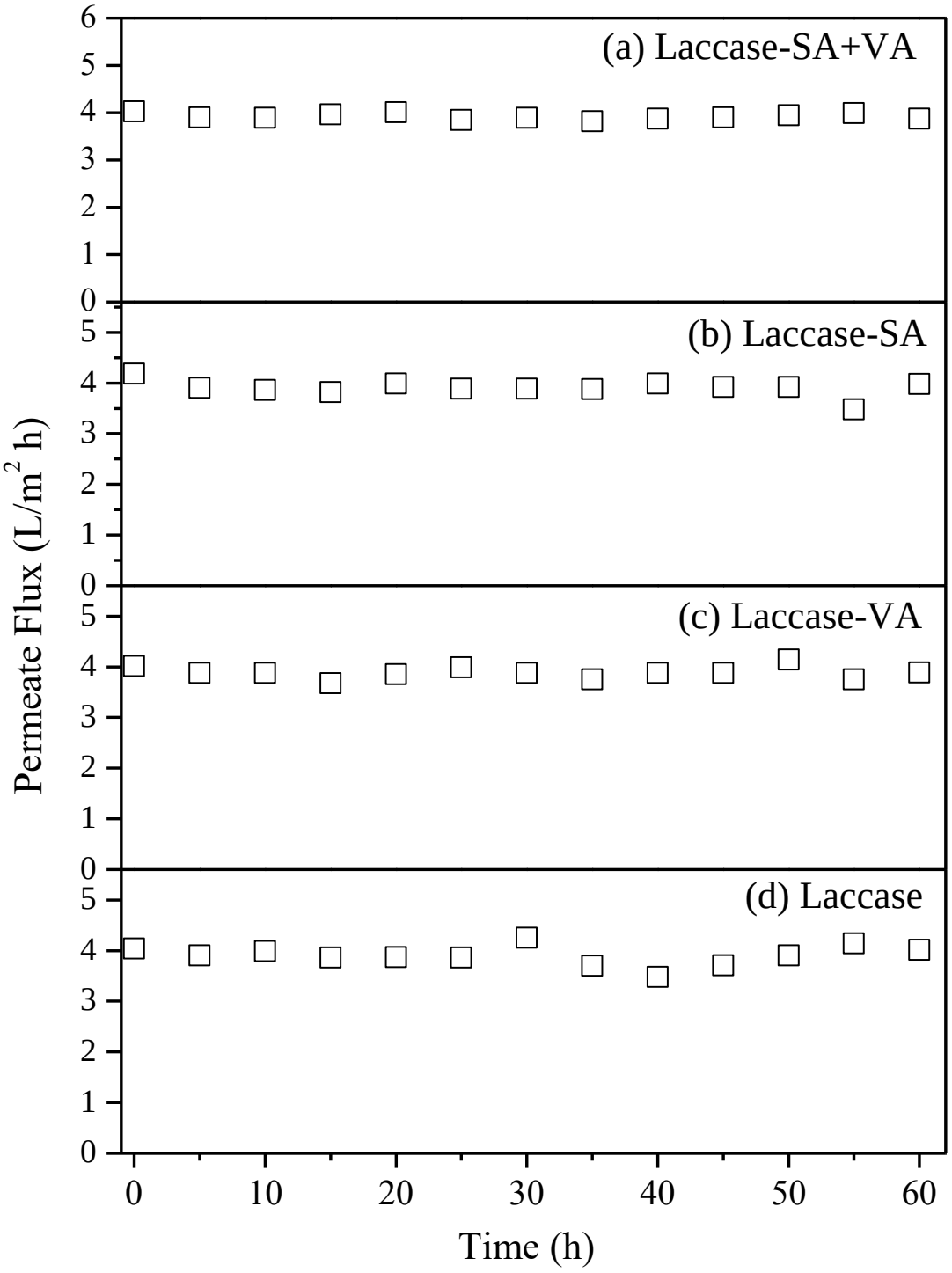
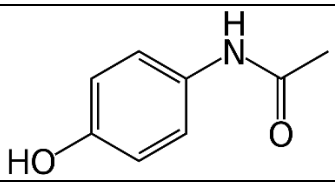
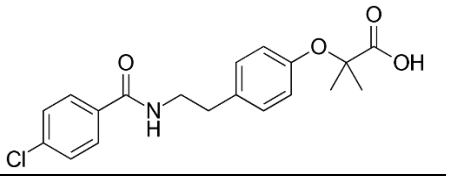
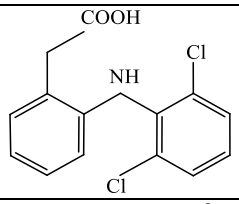
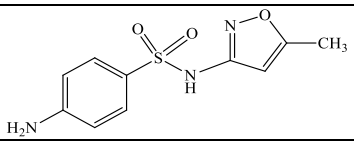
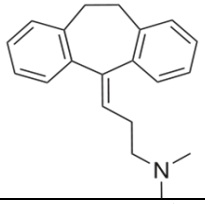
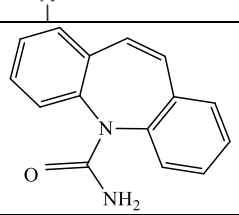
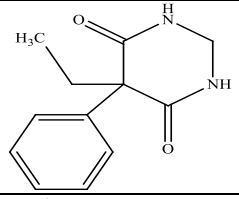
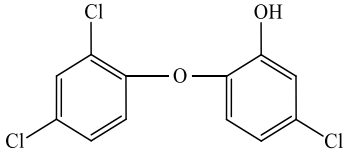
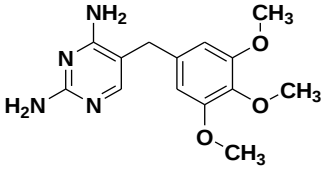
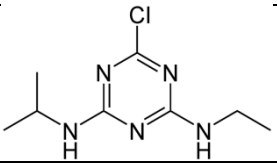


Figure 5-5. Permeate flux obtained during the long-term operation (60 h) of enzymatic membrane distillation (MD-EMR) with and without the addition of mediators. Feed and distillate temperature were controlled at 30 and 10 °C, respectively during all experiments. The cross-flow rate of both feed and distillate side was set at 1 L/min (corresponding to a cross-flow velocity of 9 cm/s).

Table 7-1. Chemical formula and structures of the selected micropollutants

Type	Name	Chemical Formula	MW (g/mol)	Chemical structure
Pharmaceuticals and personal care products (PPCPs)	Acetaminophen	$C_8H_9NO_2$	152	
	Bezafibrate	$C_{19}H_{20}ClNO_4$	362	
	Diclofenac	$C_{14}H_{11}Cl_2NO_2$	296	
	Sulfamethoxazole	$C_{10}H_{11}N_3O_3S$	253	
	Amitriptyline	$C_{20}H_{23}N$	277	
	Carbamazepine	$C_{15}H_{12}N_2O$	236	
	Primidone	$C_{12}H_{14}N_2O_2$	218	
	Triclosan	$C_{12}H_7Cl_3O_2$	290	
	Trimethoprim	$C_{14}H_{18}N_4O_3$	290	
Pesticide	Atrazine	$C_8H_{14}ClN_5$	216	

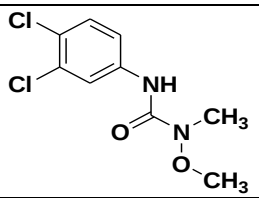
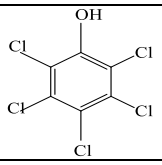
Linuron	$C_9H_{10}Cl_2N_2O_2$	249	
Pentachlorophenol	C_6HCl_5O	266	

Table 7-2: LC-MS analysis eluent gradient time program. adapted from (Xie et al., 2013)

Time (min)	Eluent B proportion (%)*
0	10
6	10
8	23
15	23
16	45
25	45
26	85
30	85
31	10
35	10

* Eluent A contains 0.1% (v/v) formic acid in Milli-Q water; eluent B is acetonitrile.